# Comparative analysis of eukaryotic (*Homo sapiens* and *Oryza sativa indica*) Class IV Sirtuins

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## Abstract

Sirtuins are a class of epigenetic modulator that are involved with essential cellular processes like metabolism, aging and genome stability. We investigate class IV sirtuins to explore the mechanistic and regulatory aspect of this protein family. To understand the role of class IV sirtuins better we took representatives from both human and plant. Human class IV sirtuins HsSIRT6 and HsSIRT7 are nuclear and nucleolar proteins, respectively whereas OsSRT1 is a nuclear protein. Using HsSIRT6 as a control/model, we investigate the biochemical and epigenetic activities of HsSIRT7 and OsSRT1. From our studies, we found that N and C terminal region of these proteins have important role in their enzymatic activities and substrate recognition. In HsSIRT7, N-terminal region is associated with protein-protein interactions and C-terminus is often associated with subcellular localization. In case of OsSRT1, the extended C-terminal region plays important role in substrate recognition. Sirtuins in general have a unique substrate specificity. There were available reports of H3K9Ac deacetylation by OsSRT1 and H3K18Ac deacetylation by HsSIRT7. Our studies shed light on its dual enzyme capability with preference for mono ADP ribosylation (mono ADPr) over deacetylation. Just like its plant counterpart, a comparison of these dual activities suggests HsSIRT7's preference for the mono ADPr transfer over its deacetylation of H3K18Ac. Several new targets for both the deacetylation and ADP ribosylation were detected. Class IV sirtuins can specifically transfer the single ADP ribose group on its substrates in an enzymatic manner. So, we were the first to discover the ADP ribosylation activity in HsSIRT7 and OsSRT1, which were previously reported as only deacetylases. This mono ADPr effect is not well known in plants, more so for deacetylases. The products of this reaction (NAM and ADP ribose) have immense negative effect on these enzymes suggesting a tighter regulation. Under different abiotic stress conditions in rice plants, we could link this ADPr activity to the DNA repair pathway by activating the enzyme PARP1. The histone specific deacetylation is also related with OsSRT1 upregulation under these stress situations. Metal toxicity in plants also influences these enzyme activities. Mono ADP ribosylation in cells is often linked to different metabolic disease conditions. This kind of modification of transcription factors, p53 and ELK4 by HsSIRT7 may play a key role in maintaining the tumor phenotype. Thus, HsSIRT7 becomes an important therapeutic hotspot for drug designing to counter several disease conditions. Screening of several chemical compounds with HsSIRT7 active site has been carried out in this study.

**Abbreviation by appearance order:**

| | |
|---|---|
| NAD | Nicotinamide adenine dinucleotide |
| ADPr | Adenosine diphosphate ribosylation |
| HDAC | Histone deacetylase |
| ELK4 | ETS transcription factor ELK4 |
| ADP | Adenosine diphosphate |
| PARP | Poly adenosine diphosphate-ribose polymerase |
| KAP1 | KRAB-associated protein-1 |
| LINE1 | Long Interspersed Element–1 |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| IPTG | Isopropyl β-d-1-thiogalactopyranoside |
| DTT | Dithiothreitol |
| TBS | Tris Buffered Saline |
| PAR | Poly ADP ribosylation |
| NHEJ | Non homologous end joining |

## (1) Introduction

Sirtuin family of proteins is quite prevalent in all organisms. They play a very important role in regulating various cellular processes ranging from metabolism to genome stability in different cellular locations. They achieve this by mainly deacetylating the acetyl lysine residues of the histone and non-histone targets in a $NAD^+$ dependent manner. This class III members of HDAC superfamily are further divided into five subclasses: I-IV and U. Among the seven human sirtuins, HsSIRT1 to 3 belong to class I, HsSIRT4 and 5 belong to class II and III, HsSIRT6 and HsSIRT7 belong to class IV (1, 2). HsSIRT1, 6 and 7 are nuclear/nucleolar, HsSIRT3, 4 and 5 are mitochondrial and HsSIRT2 is mainly a cytosolic protein. The two members, HsSIRT6 and 7 share an overall sequence identity of approximately 38%. H3K9Ac and H3K56Ac are the substrates for HsSIRT6 deacetylation (3–5) but HsSIRT7 is very specific for H3K18Ac as a lysine deacetylase target (6). The mechanism of this substrate specificity in these class IV sirtuins is not quite clear. Three-dimensional structures of most of the human sirtuins are available in apo or/and different ligand complex forms. In the case of HsSIRT7, structural information of only the N terminal region (residue range 3-73) is available (7). All the sirtuins have a phylogenetically conserved catalytic core and variable N and C terminal regions with respect to length and sequence. The terminal regions of sirtuin proteins have varied functions. The N and C terminus of yeast Hst2 are involved in homotrimer formation, which is responsible for the autoregulation of its activity (8). Among nuclear proteins, HsSIRT1 has extensions which regulate its deacetylase activity (9). There is no data available for the role of N or C terminus of HsSIRT7.

One of the major involvements of sirtuins is in tumorigenesis, thus making them very important therapeutic targets. Barber *et al.* have concluded that HsSIRT7 deacetylation is related to highly malignant cancer formation. This enzyme with the association of ETS domain-containing protein Elk-4 (ELK4) binds to promoters of various tumour suppressor genes such as Nucleoside diphosphate kinase A (NME1) and COP9 signalo some complex subunit 2 (COPS2) to bring about this action (6). HsSIRT7 is also overexpressed in human thyroid carcinoma cell lines and tissues (10). On the other hand, HsSIRT6 acts as a tumour suppressor, an over expression of HsSIRT6 leads to massive apoptosis in different cancer cells (11) along with it, HsSIRT6 has roles in various processes like DNA double strand break repair (12), metabolic pathways (13) telomerase maintenance (14) etc. HsSIRT7 has role in ribosome biogenesis (15), maintaining normal cardiac health (16). Bouras *et al.* have suggested that HsSIRT7 activates RNA Pol I-mediated transcription and regulates the expression of ribosomal RNA genes, thus it has been described to be a part of the RNA Pol I machinery (17). Kokkonen *et al.* found quercetin and three pseudopeptidic compounds as the most potent HsSIRT6 inhibitors. These are the first modulators of HsSIRT6 activity at the H3K56Ac deacetylation site which are physiologically important. No inhibitor studies for HsSIRT7 are reported till date. As there are various structural and functional data available for HsSIRT6, further work on class IV sirtuins can be done using this enzyme as a model or control.

In comparison, there are three sirtuins present in *Oryza sativa indica.* OsSRT1 belongs to class IV superfamily, having sequence identity with well-studied HsSIRT6 (46 %) and HsSIRT7 (38 %). It has 29% sequence identity with OsSRT2 (class II) and 23% with OscobB (class III).

OsSRT1 is instrumental in deacetylating H3K9Ac at the promoter site, which is linked with several metabolic processes in plants (18). RNAi analysis revealed down-regulation of OsSRT1 gene causing DNA damage and cell death under stress (18). Zhong *et al*. showed that overexpression of this gene in *Arabidopsis* leads to increased tolerance against environmental stress (19). It also has negative effect on leaf senescence by repressing the genes of methanol-jasmonate cascade (20). OsSRT1 represses glycolysis by deacetylating the OsGAPDH thus reducing its stress induced nuclear accumulation (21). However, the molecular mechanism of the OsSRT1 action in most of these cases is not clear.

One of the other post-translational modifications, ADP ribosylation is mostly linked to DNA repair, transcription and pathogenesis (22). ADP ribosylation can involve both mono as well as poly ADP ribose transfer from the $NAD^+$ molecule. In plants, this type of modification is widely studied in PARP superfamily (23). There are certain members of the human PARP superfamily like PARP3, PARP7, PARP10 etc which show mono ADPr activity (24–26). But there is no data available for this process amongst plant PARPs. Mono ADPr is a reversible chemical reaction, used to regulate different cellular processes. The role of this activity in plants is not well understood except in its bacterial pathogenesis. Here the plant pathogens secrete effector molecules, which can ADP ribosylate the host proteins involved in plant immunity, in order to promote infection (27, 28). Sirtuins even though a member of the HDAC family are known to catalyze other chemical reactions in addition to deacetylation. ADPr transfer activity was widely reported in sirtuins of several prokaryotes like *plasmodium falciparum* (29), *trypanosoma brucei* (30), *E. coli* and *salmonella* cobB (2, 31) etc. Yeast Sir2 also possessed this dual enzyme activity (32). Among human sirtuins, two of them were reported to have robust ADPr transfer activity. HsSIRT4, with very weak deacetylase activity, could downregulate glutamate dehydrogenase by ADPribosylating the enzyme (33). In 2005, it was reported that mouse SIRT6 could self-ADP-ribosylate, but no deacetylation activity could be detected for this enzyme (34). Further characterization of this enzyme mechanism was not carried out. Later, its weak deacetylase activity was reported (3, 35). HsSIRT6 could ADP ribosylate PARP1 and activate the DNA repair process (36). It could also modify nuclear repressor protein KAP1, resulting in repression of LINE1 transposable elements (37). Initially, HsSIRT7 has been found to specifically deacetylate H3K18Ac (6). Later studies suggested its histone desuccinylase activity (38). Along with our work, another group suggested the involvement of its auto-ADP ribosylation in chromatin organisation under glucose starvation (39).

Plant HDACs including sirtuins are thought to be an important regulator of different metabolic pathways under stress conditions. Their role in plants is slowly unravelling. Though, kinetic studies on these proteins are lacking. There are several unanswered questions: Does plant sirtuins have multiple enzymatic capabilities? Do they have non-histone targets as their substrates to regulate the system? How they can compensate for the role of seven human sirtuins under different cellular conditions in equally complex plant system?

**(2) Objectives (as specified in approved 1000 words)**

(1) Differentiation of molecular mechanism of class IV sirtuin catalysis.

- Determination of class IV sirtuin enzyme activity.
- Roles of N and C terminus of class IV sirtuin.
- Substrate specificity of class IV sirtuin.

(2) Analysis of the interacting partners of class IV sirtuins for better understanding specific physiological mechanism.

(3) Molecular modelling and virtual screening for inhibitors for SIRT7.

- Inhibitor design for the Sirt7 active site.

**(3) Material and Methods**

**3.1 Reagents:** Restriction enzymes, Taq DNA polymerase and T4 DNA ligase were from New England Biolabs, USA. Primers were synthesized from IDT DNA, USA. All the chemicals of analytical grade were procured from Himedia, SRL chemicals and Sigma Aldrich Co., St. Louis, MO. Antibodies used in this study are anti His (sc-8036), anti PARP2 (PHY0218S), anti H3(ab18521), anti H4 (ab177840), anti PARP1 (ab110915), anti-biotin (ab53468), anti-Poly ADPr (ab14459). rPARP1 (ab123834), acetyl Histone H3 antibody sampler kit (9927T), acetylated Histone H4 antibody sampler kit, NHEJ antibody panel (ab213658) was purchased from Abcam, USA. Bacterial plasmid expressing p53 protein was a kind gift from Dr. Smarajit Polley, Bose Institute, Kolkata, India. His-tagged-p53 was purified using the protocol supplied by them. HIS-tagged SIRT6 construct was also a kind gift from Dr. N R Sundaresan, IISc, Bangalore, India. Anti OsSRT1 antibody against GST tagged core protein was custom synthesized from rabbit (Biobharati Pvt Ltd, India). This OsSRT1 construct was purified using Ni affinity chromatography.

**3.2 Cloning, overexpression and purification of constructs:** HsSIRT7 full length construct was subcloned from EGFP-C3-HsSIRT7 construct, kindly provided to us by Dr. Ramakrishna, ILBS, Delhi, India (40). All the HsSIRT7 constructs were prepared in pET30a expression vector between *NcoI* and *BamHI* restriction sites. The OsSRT1gene was inserted in same vector between *BamHI* and *EcoRI* restriction sites. pET30a-HsSIRT7 mutants (H187Y, S111A, S115Y and R120A) and OsSRT1 mutant (H134Y) were prepared using site directed mutagenesis protocol. All the DNA sequences of the HsSIRT7 constructs were checked by a DNA sequencing service.

The proteins were overexpressed in *E. coli* BL21 (DE3) strain. The cell culture was grown @ 37 °C till the OD reached 0.7 and then induced with 1 mM IPTG and further grown overnight @ 18 °C. The cells were harvested by centrifugation at 4000 rpm for 30 min. The resultant pellet was resuspended in lysis buffer (50 mM Tris pH7.5, 300 mM NaCl, 10% glycerol, 2 mM DTT, 50 mM imidazole for HsSIRT7 and 50 mM Tris pH8, 300 mM NaCl, 10% glycerol, 2 mM DTT, 50 mM imidazole for OsSRT1). The cells were lysed using a sonicator. The cell debris were removed by centrifuging at a high speed of 14,000 rpm for 45 min. The filtered

supernatant was loaded on a pre-equilibrated HisTrap FF column (GE Healthcare, USA). The HsSIRT7 protein was eluted in 250 mM imidazole containing buffer and further treated with DNAse (2 μg/ml). Purification method for HsSIRT6 is same as HsSIRT7. The purified constructs were desalted with 25 mM Tris (pH 7.5) for HsSIRT7 and (pH 8) OsSRT1), 150 mM NaCl, 10% glycerol and 2 mM DTT for further experiments. For oligomerization analysis of His-HsSIRT7 FL and His-HsSIRT7-CC, the purified proteins were applied on an analytical size exclusion column (SEC, superdex200 10/300, GE Healthcare, USA) pre-equilibrated with buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 10% glycerol, 5 mM DTT at a rate of 1 ml/min (run by AKTA protein purification system). The purified proteins were run on SDS-PAGE and found to be more than 90% pure. The final yield of HsSIRT7 and its other constructs were within the range of 0.5–1 mg per gram of cell. OsSRT1 constructs have comparable yield from this purification process.

**3.3 Homology Modelling of OsSRT1 and HsSIRT7:** Homology model of OsSRT1(483 aa) and HsSIRT7 (402 aa) were prepared using ROBETTA and Discovery studio. HsSIRT6 (3k35) and 5iqz (N-terminus HsSIRT7) were used as templates. The models were further minimized using modeller to get most of the amino acid residues in the allowed region of Ramachandran plot parameters as analyzed in PROCHECK.

**3.4 Pulldown studies of sirtuin constructs with histones and non-histone targets:** Ni pull down experiments were performed using 1.5 μg His tagged HsSIRT7 constructs and 1 μg recombinant human Histone H3 (NEB) and mixed with Ni-NTA slurry (Qiagen). The reaction mix slurry was incubated at 4 °C for 4 h s. Then it was centrifuged at 5000g, 4 °C for 10 min. The flowthrough was collected and the beads were thoroughly washed several times with buffer (50 mM Tris buffer pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM PMSF and 1 mM DTT) and centrifuged for 10 min each. The beads were then mixed with a sample dye and boiled. The samples were then resolved using SDS-PAGE. Western blot was performed to study the different HsSIRT7 construct - H3 interactions using anti H3 antibody. Similar experiments were performed to study the interaction of different HsSIRT7 constructs and recombinant ELK4 on SDS PAGE using Coomassie staining. Similar Ni pull down experiments were carried out using His tagged OsSRT1 constructs with nuclear extract from rice leaves (histones and PARP1).

**3.5 *In vitro* lysine deacetylation activity assay:** The deacetylase reaction of purified HsSIRT7 and OsSRT1 were performed at 25 °C for 2 hr. Buffer composition for the reaction was 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM DTT and 1 mM $NAD^+$, 10% glycerol for HsSIRT7 and same buffer with pH 8.0 for OsSRT1. A 40 μl of reaction mixture was set containing 1 μg HsSIRT7 (1.5 μg for OsSRT1 experiment) and mutants, 1 μg H3 and volume was made up by buffer. The reaction was stopped by adding the sample dye and boiling it. The reaction mix was then resolved using 12% SDS-PAGE and immunoblot analysis was done by initially blocking the nitrocellulose membrane in 5% milk + TBS for 20 min s. HRP conjugated rabbit monoclonal H3K18Ac (site specific antibody of H3 and H4 for OsSRT1 experiment) antibody was used to detect the deacetylase activity of the different HsSIRT7 constructs. Using dot blot method saturation kinetics for deacetylation were performed with 0–600 μm [$NAD^+$] with a

fixed concentration of histone H3 (60 μM). Reaction mixtures were incubated at 37 °C for 30 min and contained 6 nm HsSIRT7 enzyme. All the experiments were conducted in triplicates.

**3.6 ADP ribosyl transferase activity assay:** The ADP ribosylation reaction was performed at 25 °C for 2 h. Buffer composition for the reaction was 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM DTT and 75 μM $NAD^+$ and 40 μM 6-biotin-17-NAD (Bio-$NAD^+$, R&D, Minneapolis, USA). The reaction mix containing 2 μg purified HsSIRT7 constructs/mutants were then resolved using 12% SDS-PAGE and the immunoblot analysis was done by initially blocking the nitrocellulose membrane in TBST for 30 min. Rabbit monoclonal antibiotin antibody (ab19221) was used to detect the biotinylated ADP ribose moiety of the protein. This was followed by incubation with HRP conjugated goat secondary antibody and developed with ECL solution (BIORAD). This experiment was further repeated with recombinant ELK4 (2 μg), PARP1 (2 μg) and p53 (2 μg) as substrates for HsSIRT6 and HsSIRT7 and with recombinant Histones (NEB) (0.5μg) and PARP1 (5μg) as substrates for OsSRT1. ADPr activity has also been examined in presence of 0.5 mM concentration of various salts ($CuSO_4$, $ZnCl_2$, $FeCl_3$, $MgCl_2$, $MnCl_2$ and $CaCl_2$). The intensity of bands in the blots were analyzed using ImageJ software (NCBI) (41). For dot blot analysis, 6 nM HsSIRT7/OsSRT1 mixed with varied concentrations of Bio-$NAD^+$ (0-100 μM). For the determination of kinetic parameters of H3 ADPr reaction, constant conc. of H3 (3.3nM) was added to the same kind of reaction mix. All the experiments were carried out in triplicates.

**3.7 Determination of kinetic parameters using Dot Blot Analysis:** The reaction mixtures were prepared and carried out as mentioned above. The finished reactions were desalted to remove the unused Bio-$NAD^+$ or other buffer constituents. The reaction mix was then dot blotted on a nitrocellulose membrane and air dried. The blots were developed as in western blot protocol. The standard curve of Bio-$NAD^+$ was also prepared for the said reaction. The intensity of the dots was analyzed using ImageJ software (NCBI) and Microsoft Excel. Saturation kinetics using Non-Linear regression (Michaelis Menten Model) was prepared using GraphPad Prism version 9.1.1 (San Diego, USA).

**3.8 Fluorometric assay:** Intrinsic Tryptophan fluorescence emission studies of 1 μM HsSIRT7, its different constructs and H187Y mutant with $NAD^+$ and its analogs were conducted on a plate reader (Biotek SYNERGY H1 microplate reader). Samples were excited at 295 nm and emission spectra was monitored from 320 to 420nm. Experiments under native conditions were performed in 50 mM Tris, pH 7.5, at room temperature. Increasing concentrations of $NAD^+$ and its analog from 0 to 600 μM were used. Binding constants were calculated using one site binding equation in GraphPad Prism version 8.3.0 (San Diego, USA).

## (4) Results and Discussion

### (1) Differentiation of molecular mechanism of class IV sirtuin catalysis

- **Determination of class IV sirtuin enzyme activity**

Most of the class III HDACs have multiple catalytic abilities. Besides H3K18Ac deacetylation, we detected the transfer of Bio-ADPribose from $NAD^+$ to HsSIRT7, thus confirming auto-

ADPribosyl transferase activity. Mutating the conserved catalytic base $His^{187}$ to Tyr (H187Y) prevented this ADPr transfer **(Fig1A)**. We also checked whether it is capable of transferring a single or multiple units of ADPr. Western blot using poly ADPr antibody has been executed which showed no bands confirming its mono- ADPr transfer reaction. As PARP1 has been widely characterised for its poly ADPr activity, we have used this enzyme as a positive control in this experiment **(Fig1B).** HsSIRT7 does not ADP ribosylate histones (H3 and H4). Through dot blot technique we have calculated the rate of the auto- ADPr transfer activity as 0.39 ± 0.03 μM/min ($k_{cat}$ = 65 ± 5 $min^{-1}$) with $K_m$ of 36.1 ± 6 μM for Bio-$NAD^+$ whereas HsSIRT7 mediated H3K18Ac deacetylation has a lower $k_{cat}$ value of 3.6 ± 0.15 $min^{-1}$ **(Table1)**. Similarly in OsSRT1, besides deacetylation of H3K9Ac, we could detect its ADPr activity. The recombinant protein (1μg) can fully ADP ribosylate itself within a timespan of 1hour. For this it requires $NAD^+$ in the reaction mix. The H134Y catalytic mutant was not able to ADP ribosylate itself. In plants, auto ADPr activity of deacetylase enzymes have not been reported yet. Just like HsSIRT7, this enzyme is also capable of transferring a single moiety of ADPr on itself. Once it is established that these proteins can mono ADP ribosylate, we focussed on the amino acid residues that can get modified in this process. The auto-modification of HsSIRT7 using chemical stability assay detects its ADP ribosyl linkage with Arg and Cys residues **(Fig1C)**. Reagents like $HgCl_2$, NaCl and $NH_2OH$ were used to detect the breakage of covalent linkage of ADP ribose unit with Cys, Lys and Arg residue, respectively (42–44). Interestingly among other human sirtuins, HsSIRT4 is a Cys specific ADP ribosylase (33) and HsSIRT6 is specific for Lys residues (36). In case of OsSRT1, chemical stability assay of this enzyme's auto ADPr revealed that the transfer is mostly targeted to Arg residues of the substrate. It is also seen that this ADPr reaction in an enzymatic manner **(Fig1D)**.

- **Roles of N and C terminus of class IV sirtuin**

Based on the protein sequence alignment of HsSIRT7 with its homolog HsSIRT6, it has extra region (1-90) on N- and (330-400) on C-terminus of this enzyme. The homology model of 47 kDa HsSIRT7 protein reveals the conserved catalytic base $His^{187}$ of HG motif in the 275 aa core region. The extra N terminal region forms a long, flexible helical hook like structure. We hypothesize that this region may change conformation on substrate binding. Here, the catalytic core contains the large domain with Rossmann fold for $NAD^+$ binding separated by a small zinc binding domain of three antiparallel strands. Similarly, in OsSRT1 there are two distinct areas for active site binding: the $NAD^+$ (Rossmann Fold) and the other is for the $Zn^{2+}$, there is also a huge C terminal region (290-483) not present in the other classes of plant sirtuins, forming a separate domain. The function of this domain is unclear based on its folds but can be speculated as a regulatory site or accessory protein binding site for this enzyme. All these structural features contribute for its substrate specificity. **(Fig2-4)**

In order to understand the possible roles of N- and C- terminal regions of class IV sirtuins with respect to various processes like binding to substrates and/or accessory proteins, catalysis and/or oligomerization, we have created six constructs each of HsSIRT7 and OsSRT1 protein: full length (FL: 1-400 and 1-483), catalytic core region (CC: 91-330 and 46-289), N-terminus with CC region (ΔC1:1-330 and 1-289), N-terminus alone (ΔC2:1-90 and 1-45), C-terminus

with CC region (ΔN1:91-400 and 46-483), and C-terminus alone (ΔN2:331-400 and 290-483). **(Fig5)**

In *Pf*Sir2 (45) and yeast Hst2 (8), these terminal regions are essential for the oligomerization of the protein. Our size exclusion chromatography experiments have concluded that N and C termini of HsSIRT7 as well as OsSRT1 are not involved in oligomerization. In these SEC analyses the HsSIRT7 FL and the CC construct, that lacks both the N and C- terminus, eluted as a monomer. Similarly, OsSRT1 also eluted as a monomer. Further, Ni pull down experiments were performed to study the binding of ligands with the abovesaid HsSIRT7 and OsSRT1 constructs. We observed that the full length (FL) and catalytic core (CC) in addition to the ΔC1 construct could bind to both histone H3 and accessory protein ELK4, and PARP1. ΔC2 in HsSIRT7 bound weakly with the ligands and showed almost no activity as it also lacks the catalytic core region. ΔN1 and ΔN2 constructs on the other hand did not bind to any of these ligands. Though there have been some weak interactions between the ΔN1 construct and substrates as it showed deacetylase activity with a rate of $0.39 \pm 0.1$ μM/min. Dot blot analysis found comparable activity for FL and ΔC1 construct with little weaker activity for CC. Also, catalytic core region (CC) showed almost two fold lower $K_m$ value than the FL for $NAD^+$ **(TABLE1 & Fig6)**. These values suggest the importance of N-terminus in substrate binding. From all these studies, the role of C terminus is not quite clear in HsSIRT7. An earlier report by Kiran *et al.* suggested that the C terminus contains the NoLS signal ($K^{392}RTKRKK^{398}$) for transportation of HsSIRT7 to nucleolus. Mutation of these residues abrogated HsSIRT7's localization to nucleolus. A region (197-391) of HsSIRT7, which is devoid of N- and C terminus, remained in the cytoplasm (46). So assumption can be made that the C-terminus preferably has the nucleolar localization role. This is similar to the C-terminus region of HsSIRT6 that also contains the NLS signal (47). Full length HsSIRT7 with 38% sequence identity, shares 37% and 30.5% identity with the N- and C-terminus of HsSIRT6, respectively. Sequence alignment of several metazoan HsSIRT7 proteins revealed the conservation in both the N- and C-terminal regions. This is in contrast to HsSIRT6 where the N-terminus is majorly conserved. The only exception is HsSIRT7 in *D. melanogaster*, which has an extra 371 amino acid residues at its C terminus. This finding suggests a critical role for both N- and C- terminus in HsSIRT7 proteins **(Fig7A)**. In case of OsSRT1, very short N terminus (~45 aa) and longer C terminus (~232 aa) region has been found to be involved in ligand binding, using Ni pull down assay. ΔC2 binds very weakly with the substrates H3 and PARP1. Presence of acidic residues at this end may be attracting the basic histones. There is a possibility that presence of both the terminus stabilizes the binding of ligands with the catalytic core region. Since CC alone showed the lowest catalytic efficiency with high $K_m$ value. Even though OsSRT1 has 46% sequence identity with HsSIRT6 we could not locate the NLS region in the C terminus of this plant protein by sequence alignment. FindMod analysis also detects PTM sites at the C-terminal end of this protein **(TABLE2 and Fig7B)**.

- **Substrate specificity of class IV sirtuins**

  Sirtuins undergo a bisubstrate reaction. They catalyse the hydrolysis of $NAD^+$ molecule coupled with protein deacetylation. Earlier reports on human sirtuins showed that in the case

of HsSIRT1 and HsSIRT2, $NAD^+$ cannot bind in the absence of an acetylated peptide (48, 49) whereas HsSIRT6 could bind directly with $NAD^+$ (35). Here, we have studied the binding abilities of HsSIRT7 with $NAD^+$ and its analogs, taking advantage of the Trp residues ($Trp^{126}$, $Trp^{251}$, $Trp^{281}$, $Trp^{300}$) at the active site. Using this intrinsic tryptophan fluorescence effect, we saw an emission peak at 340nm when HsSIRT7 FL sample was excited at 295nm. There was a decrease in fluorescence signal on $NAD^+$ binding ($K_d$ = 30±5μM). $NADP^+$ could also bind to HsSIRT7 to a certain extent with $K_d$ = 75±2 μM. However, we detected no binding when NADH was titrated into HsSIRT7 sample, suggesting that HsSIRT7 folds cannot accommodate the changes in the nicotinamide ring structure of NADH. Similarly, deacetylase activity of OsSRT1 with different analogues of $NAD^+$ (NADH, $NADP^+$ and thio-$NAD^+$) suggests specificity for mainly $NAD^+$. Fluorometric analysis of OsSRT1 also suggests binding of $NAD^+$ in absence of acetylated protein.

Ni pulldown experiments suggest the interaction of HsSIRT7 with histones H3 and H4. It is known that HsSIRT7 is very specific for H3K18Ac as a deacetylation substrate (6). Histone H4 are not the substrates for HsSRT7 deacetylation. Besides its auto ADPr, HsSIRT7 cannot ADPribosylate histone H3 and H4 whereas HsSIRT6 can ADPr H3. OsSRT1 also being a nuclear protein can interact with histones H3 and H4 **(Fig8A)**. Other than H3K9Ac, OsSRT1 can deacetylate $lys^{18}$ and $lys^{56}$ positions of H3 and $lys^5$, $lys^{12}$ and $lys^{16}$ of H4. From our kinetics data, it seems that OsSRT1 is very efficient in deacetylating H3K9Ac, followed by H3K18Ac. In case of H4, OsSRT1 can most efficiently deacetylate H4K16Ac **(Fig8B & 8C)**. In addition to its own ADPr, it can specifically modify the histone H3 and H2A but not H4.

**(2) Analysis of the interacting partners of class IV sirtuins for better understanding specific physiological mechanism.**

HsSIRT7 can interact with both H3 and H4. But they were not the targets for the ADP ribosylation and to some extent deacetylation activity of HsSIRT7. We further searched for nuclear non-histone substrates for this activity. HsSIRT7 could physically interact with both ELK4 (6) and p53 (50) and transfer the biotinylated-ADP ribose to these transcription factors. HsSIRT7 mutant (H187Y) was unable to ADP ribosylate both these targets. **(Fig9A&9B)** The significance of ADPr modification of ELK4 by HsSIRT7 is not quite clear as there is limited information available regarding this transcription factor. ELK4 is mostly implicated in prostate cancer studies (51). Under certain cellular conditions, this might be a regulatory mechanism for controlling the deacetylation of H3K18Ac as ELK4 recruits HsSIRT7 to the sites of deacetylation. Alternatively, it is possible that the ADP ribosylation of ELK4 augments its involvement in cancer metabolism. Another nonhistone target in the nucleus that HsSIRT7 could equally transfer the ADP ribose is PARP1. PARPs and sirtuins are known to interplay in influencing the $NAD^+$ pool in the cell as both utilize this coenzyme for their activities **(Fig9C).** PARPs are also known to influence sirtuins (52). The transfer of ADP-ribose on p53, ELK4 and PARP1 are specific to HsSIRT7 as we did not see the transfer of biotin-ADPr on these substrates by HsSIRT6.

Ni pull down experiments of OsSRT1 with nuclear extract from rice leaves capture its interaction with DNA repair machinery (PARP1, PARP2, H2A.X, Ku70, Ku80 and

gammaH2A.X) under different abiotic stress conditions. This is in addition to the interactions with histones. Interestingly, OsSRT1 is able to ADPr the two main players of DNA damage pathway, OsPARP1 and OsPARP2. This modification of PARP1 significantly increases its own PAR activity. **(Fig10)**

**(3) Molecular modelling and virtual screening for inhibitors for SIRT7.**

HsSIRT7 has extended N terminal loop region, OsSRT1 has extended C terminus region. These regions contribute to its interaction with other proteins and biochemical activities. To further understand the biochemical mechanism in class IV sirtuins we ran virtual screening to identify inhibitors. For this, we used $NAD^+$ binding site of a probable HsSIRT7 tertiary structure model as a control **(Fig11A)** which was designed based on HsSIRT6 crystal structure.

We downloaded the fifty HDAC inhibitors from Pubchem and ZINC database **(TABLE3)** and docked it with HsSIRT7 model, which was generated using Discovery studio software. Using PyRx virtual screening tool (Autodock Vina) all the inhibitors were simulated against the complete HsSIRT7 model. We further manually visualised the docking results, with a hope to identify the region for inhibitor binding. From the experiment we have found that $GLN^{299}$, $SER^{270}$, $PHE^{239}$, $ALA^{108}$, $ARG^{120}$, $TYR^{119}$, $CYS^{169}$, $LEU^{216}$, $ILE^{236}$ along with $HIS^{187}$ in some cases forms the inhibitor binding tunnel in HsSIRT7. Substance 9804992 [Panobinostat (LBH589)] from Pubchem database showed the highest binding affinity with HsSIRT7 (-10.1kcal/mol) **(Fig11B)** and substance 11538455 [Pracinostat (SB939)] shows a little less affinity (-9.1kcal/mol) with the protein **(Fig11C)**. Other substances with lesser affinity show a slight shift in binding site. Sirtuin effectors like Trichostatin A [Pubchem number 44732] with a binding affinity of -8.1kcal/mol **(Fig11D)**, sodium butyrate [Pubchem number 6918837] with a binding affinity of -7 kcal/mol **(Fig.11.E)** and HDAC effector like Dacinostat [Pubchem number 49855250] with a binding affinity -8.3 kcal/mol **(Fig11F)** binds to a site that composed of $CYS^{169}$, $VAL^{237}$, $SER^{269}$, $ARG^{120}$, $ARG^{279}$, $VAL^{125}$, $TYR^{119}$ along with $LEU^{271}$, $TRP^{126}$, $LEU^{216}$ and $HIS^{238}$. Trichostatin A binds to $HIS^{238}$ so does Panobinostat. Again, Pracinostat binds to $SER^{270}$ and $CYS^{169}$ while Sodium butyrate also binds to $CYS^{169}$ and Trichostatin A binds to $SER^{270}$. Incidentally, $NAD^+$ also binds to $SER^{270}$. So from these results we can see that these best scoring compounds interact with the residues which are also involved with $NAD^+$ binding. So, these substances can essentially block the region from $NAD^+$ binding, thus inhibiting the biochemical activity of the protein.

**(5) Conclusion**

In summary, we provide information on the structural features of HsSIRT7 and its mechanistic role in biological functions. Along with the catalytic core, the N-terminus of HsSIRT7 is required for binding to substrates. The C terminus will be involved in its nucleolar localization. Both extensions can be sites for protein modifications. Mutations in the enzyme catalytic core lead to weak binding of the substrate and subsequent loss of its function. $S^{111}$, $S^{115}$, $H^{187}$ play a significant role in its ADPr transfer activity as the mutants S111Y, S115A, H187Y mutants completely/partially lose their activity. Product inhibition of HsSIRT7 can regulate the $NAD^+$ pool in the cell. HsSIRT7 can bind to $NAD^+$ tightly without the acetylated peptide so it can also act as an independent $NAD^+$ sensor. It is known that HsSIRT7 is actively linked to

tumorigenesis so all these data will be greatly helpful in controlling this enzyme. Self-modification of HsSIRT7 can also affect the interaction of this enzyme with other regulatory partners under different stress conditions. Thus, determining the role of HsSIRT7 in these situations. This may also affect its role in different cellular mechanisms leading to tumorigenesis. We have shown that HsSIRT7 can physically interact and ADP ribosylate transcription factors like p53 and ELK4 *in vitro*. These transfer catalyzed by HsSIRT7 is very specific, as HsSIRT6 could not transfer the ADPr moiety on these proteins. Cellular relevance of mono ADPribosylation of these transcription factors needs to be further explored. In reference to Gu *et al* review, the ADP ribosylation sites for p53 are $E^{258}$, $E^{259}$ and $E^{271}$, which are located in its DNA binding domain (53). Poly ADP ribosylation of p53 has affected its DNA binding ability (54, 55). Alterations in p53 structure especially the DNA binding region leads to development of cancer (56). So, we can speculate that mono ADP ribosylation of p53 by HsSIRT7 will evidently affect its DNA binding property under stress condition, thus relating it to tumorigenesis. This can be a way by which HsSIRT7 can regulate p53 functions, that stabilizes the tumor phenotype. ADP ribosylation of PARP1 can promote it to recruit other proteins at the site of DNA damage. In support of this fact, our studies showed that HsSIRT7 can increase the PARP1 activity (approx. 53%) by ADP ribosylating it. The two nuclear proteins, HsSIRT1 and HsSIRT6, are deeply linked to the DNA damage repair processes. Though, they follow different mechanisms to influence this pathway. We know that HsSIRT7 is also linked to DNA repair in varied ways. It needs to be determined under what cellular stress conditions HsSIRT7 influences PARP1 activity.

Nuclear OsSRT1 is almost ubiquitously present in the major tissues of the plant. OsSRT1 seems to have deacetylase substrate specificity of both HsSIRT6 and HsSIRT7. The mono ADPr activity of OsSRT1 for itself as well as other nuclear proteins in plants are also reported. This sirtuin possesses dual enzyme activity similar to its human homologs. We also see a similar trend in case of OsSRT1, with a weaker deacetylase activity compared to its ADPr activity. The structural features of this plant sirtuin is highlighted with a separate C-terminal domain. This region is required for substrate binding as well as its catalysis. Metal ions like $Cu^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ have an inhibitory effect on this action suggesting a big impact on epigenetic regulation in plants under metal stress. ADP ribosylation is known to play a role in regulation of DNA damage and repair pathway (22). This kind of ADPr reaction may be reversible process as there is existence of eraser proteins in plants (57). OsSRT1 can interact directly with histones and the components of DNA repair pathway as evidenced from the Ni pull down experiments. Both PARP1 and PARP2 proteins are involved in DNA repair process in plants (58, 59). This mono ADPr action is involved in regulating OsPARP1's activity in a positive way. In addition, it is known in mammals that histone ADP ribosylation is also induced by DNA strand breaks (60). These effects play an important role in metabolism and disease conditions. Similarly, we may speculate here that OsSRT1 action on histones H3 and H2A may be in response to the DNA damage in plants under stress conditions. Overall, this ADPr study reveals an important aspect of OsSRT1 action in chromatin regulation in plant stress responses. There is also a correlation between the upregulation of OsSRT1 and deacetylation of the specific histone positions under various abiotic stress conditions. With the elucidation of mono

ADP ribosylation in plant proteins, it opens up the avenues to understand the mechanism of adaptation or tolerance under unfavourable conditions.

**(6) Significance and Future Direction of this work**

Other than deacetylation, mono ADP ribosylation is also an important post translational modification to regulate different cellular processes. This enzyme activity is deeply linked to controlling cell proliferation [57]. Though sirtuins' link with cancer through ADP ribosylation is not established, this can be an area which can be further explored. As we understand HsSIRT7's regulatory mechanism much better now so our next step will be designing of small molecules which can block this enzyme activity. $NAD^+$ sensor HsSIRT7 regulates gene activity by interacting with ELK4. We can also develop small molecules which can disrupt these interactions (PPI). This will help researcher to prevent its involvement in the maintenance of tumor phenotype, as *in vivo* studies have shown the reversal of transformed phenotype of cancer cells on inactivation of HsSIRT7. ADP ribosylation has essential role in DNA damage and repair pathway in plant, as OsSRT1 can directly interact with histones and non-histone proteins from DNA repair pathway, it impacts in epigenetic regulation in plant.

There are still several unanswered questions. In the case of HsSIRT7, is this self-modulation in response to certain cell signals? As mutation studies suggest that deacetylation and mono ADPr transfer activities are two independent processes in the case of HsSIRT7, what kind of cellular signals prompt these activities? As class IV sirtuins are showing dual enzymatic activities, a question can be asked, how is the dual activity regulated in sirtuins harboring both enzymatic activities? Or what makes one activity more important than the other for a specific substrate? In case of OsSRT1, as epigenetic regulations also involve covalent modifications in DNA or RNA, does OsSRT1 has the capability to ADP ribosylate these molecules? With this ability in its arsenal can plant also withstand the biotic stress created by bacterial or fungal pathogens? Also, we see that in case of HsSIRT7, the ADPr transfer reaction can happen in absence of any acetylated protein. Is there still any relation between the two chemical reactions?

Recent studies reveal that there are plenty of enzymes like human PARPs (PARP3, PARP4, PARP10 etc), bacterial toxins and sirtuins from Salmonella, E. coli (class III sirtuins), PfSIR2 and human HsSIRT4 (class II sirtuin), HsSIRT6 which perform mono ADPr reaction. So HsSIRT7 and OsSRT1 are new additions to this ever-growing list of mono ADP ribosyl transferases.

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**TABLE1: A summary of the Michaelis Menten parameters for the indicated HsSIRT7 constructs for its deacetylase activity.** Kinetic parameters of $NAD^+$ as a substrate for HsSIRT7 deacetylase activity of H3K18Ac was measured using dot blot method. Reactions were carried out with 0-600 µM $NAD^+$ with constant concentration of H3 (60µM). For certain HsSIRT7 constructs and mutants there was no activity. N.D. Not detected. The kinetic parameters for the HsSIRT7 dual activity using Bio-$NAD^+$ are also reported. ± indicate S.D; n=3

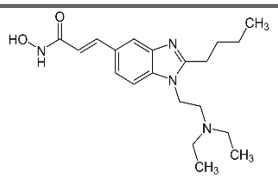
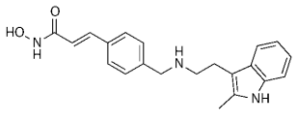
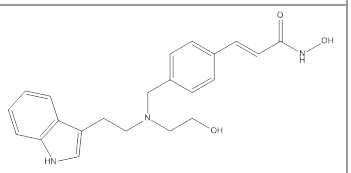
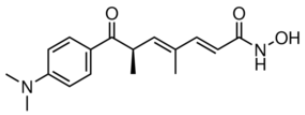
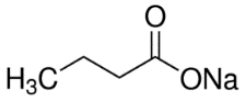
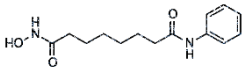
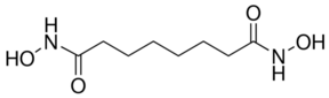
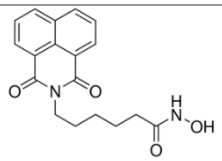
| SIRT7 constructs | $K_m$ for $NAD^+$(µM) | $k_{cat}$ ($min^{-1}$) |
|---|---|---|
| SIRT7 FL | 195 ± 20 | 3.2 ± 0.13 |
| SIRT7cc | 280.7 ± 42 | 2.2 ± 0.15 |
| ΔC1 | 373 ± 49 | 2.7 ± 0.17 |
| ΔC2 | N.D. | N.D. |
| ΔN1 | 72.4 ± 20 | 0.99 ± 0.17 |
| ΔN2 | N.D. | N.D. |
| H187Y | N.D. | N.D. |
| S111Y | N.D. | N.D. |
| S115A | 45 ± 23 | 0.97 ± 0.11 |
| R120A | 238 ± 29 | 3.1 ± 0.16 |

| SIRT7 enzyme activity | $K_m$ for Bio-$NAD^+$(µM) | $k_{cat}$ ($min^{-1}$) |
|---|---|---|
| H3K18Ac deacetylation | 253 ± 25 | 3.6 ± 0.15 |
| ADP-ribosylation | 36.1 ± 6 | 65 ± 5 |

**TABLE2: A summary of the Michaelis Menten parameters for the indicated OsSRT1 constructs for its deacetylase activity.** Kinetic parameters of $NAD^+$ as a substrate for OsSRT1 deacetylase activity of H3K9Ac was measured using dot blot method. Reactions were carried out with 0-600 µM $NAD^+$ with constant concentration of H3 (60µM). For certain OsSRT1 constructs, there was no activity. N.D. Not detected. The kinetic parameters for the OsSRT1 dual activity using Bio-$NAD^+$ are also reported. ± indicate S.D; n=3

| OsSRT1 constructs | $K_m$ for $NAD^+$(µM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| OsSRT1 FL | 168 ± 14 | 4.9 ± 0.16 | 1.76 x $10^6$ |
| OsSRT1_cc | 256 ± 21 | 2.059 ± 0.076 | 0.48 x $10^6$ |
| ΔC1 | 299 ± 43 | 1.2 ± 0.084 | 0.24 x $10^6$ |
| ΔC2 | N.D. | N.D. | N.D. |
| ΔN1 | 175 ± 27 | 3.7 ± 0.22 | 1.27 x $10^6$ |
| ΔN2 | N.D. | N.D. | N.D. |

**TABLE3**

| Number | SIRT list of inhibitors and activators | Binding energy (Kcal/mol) for HsSIRT7 | Binding energy (Kcal/mol) for HsSIRT6 | Chemical Structure |
|---|---|---|---|---|
| **1** | PRACINOSTAT (SB939) | -10.1 | -8.6 |  |
| **2** | PANOBINOSTAT (LBH589) | -9.1 | -9.7 |  |
| **3** | DACINOSTAT (LAQ824) | -8.5 | -9.4 |  |
| **4** | TRICHOSTATIN A | -8.1 | -7.9 |  |
| **5** | SODIUM BUTYRATE | -7 | -8 |  |
| **6** | SAHA | -7.8 | -8.4 |  |
| **7** | SBHA | -3.9 | -4.2 |  |
| **8** | SCRIPTAID | -7.5 | -11 |  |

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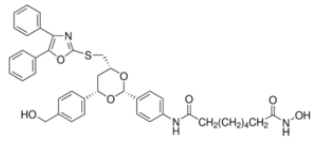
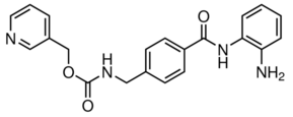
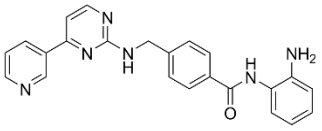
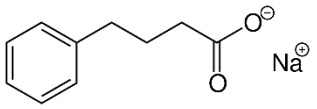
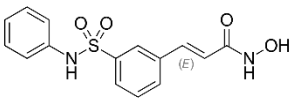
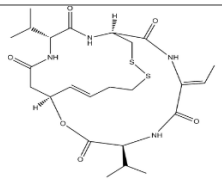
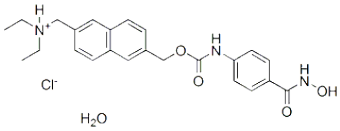
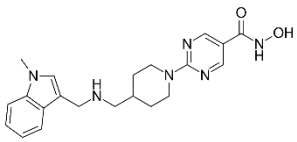
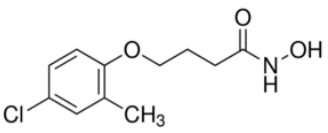
|---|---|---|---|---|
| **9** | TUBACIN | -5.8 | -6.8 |  |
| **10** | ENTINOSTAT (MS-275) | -6.8 | -6.3 |  |
| **11** | MOCETINOSTAT (MGCD0103) | -7.4 | -9 |  |
| **12** | 4-PHENYLBUTYRIC ACID (4-PBA) | -7.3 | -8.1 |  |
| **13** | BELINOSTAT (PXD101) | -7.8 | -7 |  |
| **14** | ROMIDEPSIN (FK228, DEPSIPEPTIDE) | -6.7 | -7.9 |  |
| **15** | GIVINOSTAT (ITF2357) | -8.8 | -10 |  |
| **16** | QUISINOSTAT (JNJ-26481585) 2HCL | -8.6 | -9.3 |  |
| **17** | DROXINOSTAT | -8.6 | -7.8 |  |

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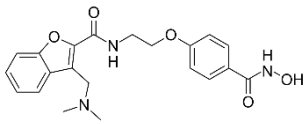
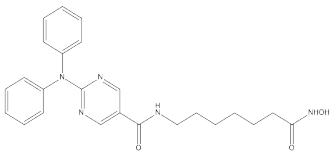
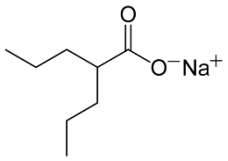
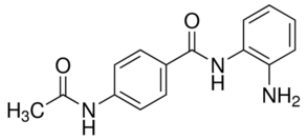
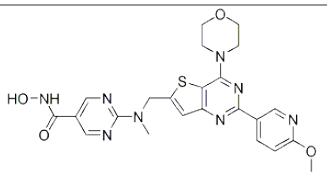
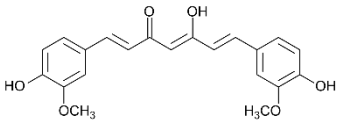
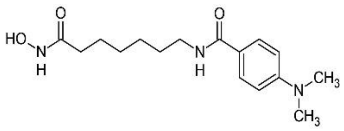
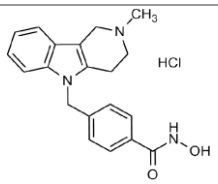
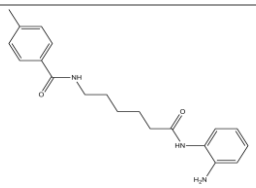
|---|---|---|---|---|
| **18** | ABEXINOSTAT (PCI-24781) | -7.6 | -8.4 |  |
| **19** | RICOLINOSTAT (ACY-1215) | -7.7 | -6.9 |  |
| **20** | VALPROIC ACID (NSC 93819) SODIUM SALT | -6.6 | -7.1 |  |
| **21** | TACEDINALINE (CI994) | -6.4 | -9.1 |  |
| **22** | FIMEPINOSTAT (CUDC-907) | -8 | -7.9 |  |
| **23** | CURCUMIN | -7.5 | -4.7 |  |
| **24** | M344 | -3.9 | -5.1 |  |
| **25** | TUBASTATIN A HCL | -5 | -6.7 |  |
| **26** | RG2833 (RGFP109) | -6.5 | -7.4 |  |

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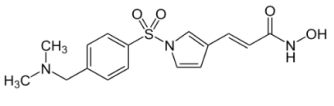
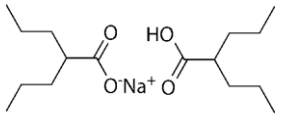
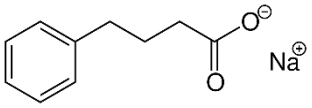
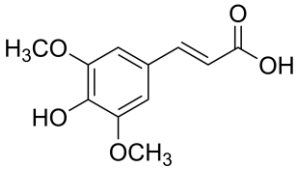
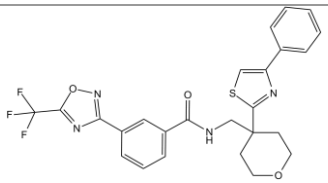
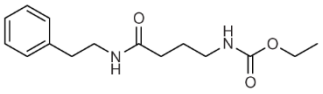
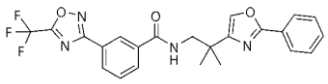
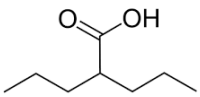
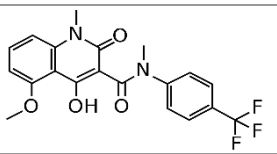
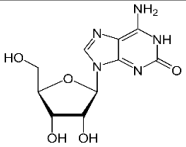
|---|---|---|---|---|
| **27** | RESMINOSTAT | -6.9 | -8.4 |  |
| **28** | DIVALPROEX SODIUM | -6.8 | -8.1 |  |
| **29** | SODIUM PHENYLBUTYRATE | -7.5 | -8.7 |  |
| **30** | SINAPINIC ACID | -8.6 | -10.6 |  |
| **31** | TMP269 | -8.8 | -6.6 |  |
| **32** | SANTACRUZAMATE A (CAY10683) | -7.2 | -10.8 |  |
| **33** | TMP195 | -8.4 | -8.3 |  |
| **34** | VALPROIC ACID (VPA) | -6.8 | -9.9 |  |
| **35** | TASQUINIMOD | -8.5 | -7.7 |  |
| **36** | ISOGUANOSINE | -8.8 | -8 |  |

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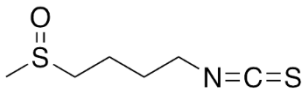
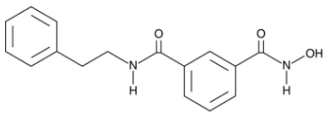
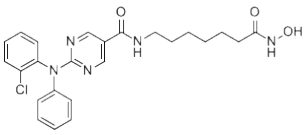
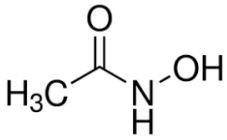
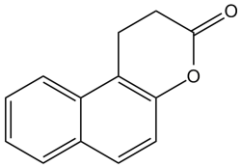
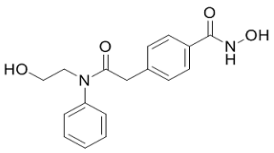
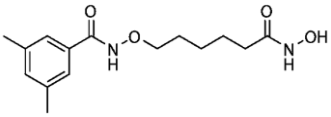
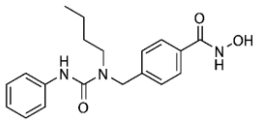
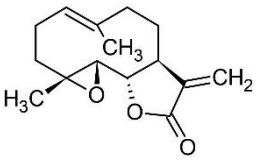
|---|---|---|---|---|
| **37** | SULFORAPHANE | -7.1 | -9.4 |  |
| **38** | BRD73954 | -8.8 | -8.9 |  |
| **39** | CITARINOSTAT (ACY-241) | -8.5 | -9.2 |  |
| **40** | SUBEROHYDROXAMIC ACID | -7.5 | -10.9 |  |
| **41** | SPLITOMICIN | -8.4 | -11.8 |  |
| **42** | HPOB | -9.4 | -8.6 |  |
| **43** | LMK-235 | -9.7 | -8.9 |  |
| **44** | NEXTURASTAT A | -7.8 | -8.1 |  |
| **45** | (-)-PARTHENOLIDE | -7.1 | -10.4 |  |

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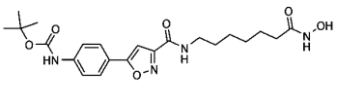
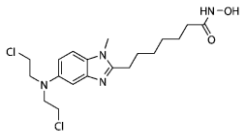
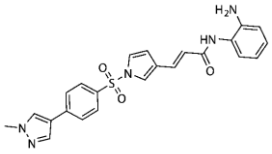
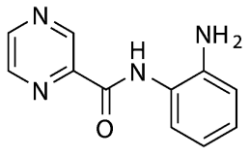
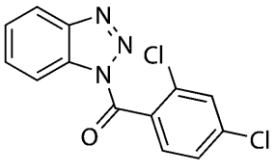
| **46** | CAY10603 | -7.7 | -9.8 |  |
| **47** | TINOSTAMUSTINE (EDO-S101) | -8.2 | -7.4 |  |
| **48** | DOMATINOSTAT (4SC-202) | -9 | -8.5 |  |
| **49** | BG45 | -7.1 | -6.7 |  |
| **50** | ITSA-1 (ITSA1) | -8.6 | -8.1 |  |

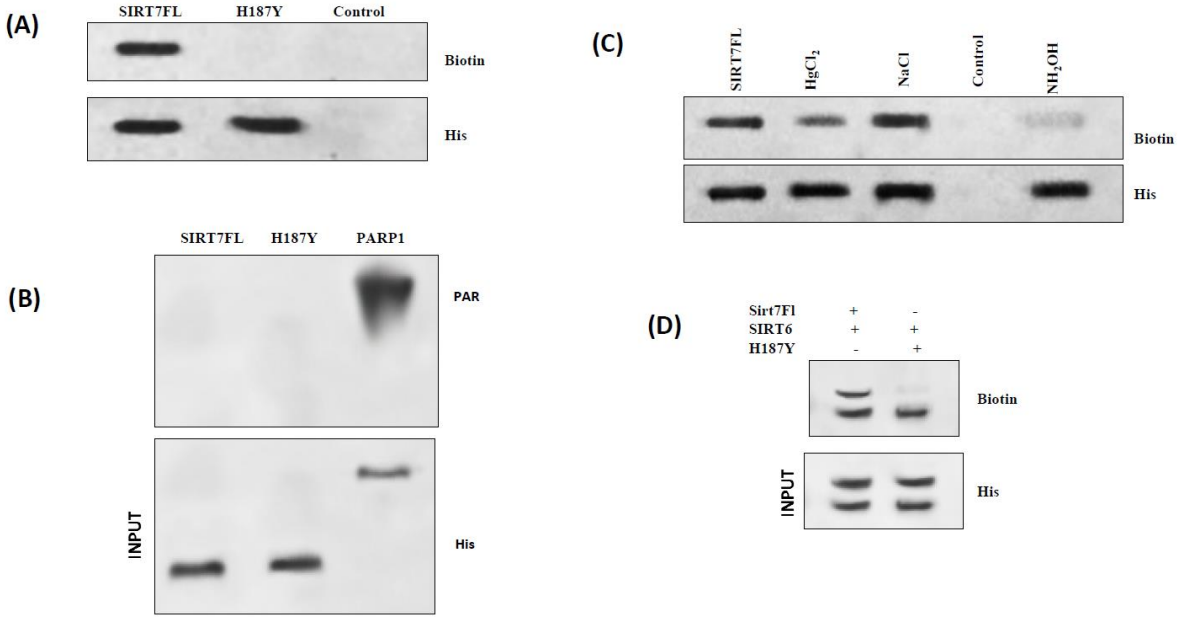



**Figure 1: Mono-ADPribosylation activity of HsSIRT7:** Here the negative control is H187Y mutant or empty plasmid (control). The loading control, developed with antiHis antibody, is shown in the lower panel. For western blot, anti-biotin antibody was used (A) the blot showed that HsSIRT7 was able to ADP-ribosylate itself using substrate Bio-$NAD^+$ (40 μM). H187Y mutant did not show this transfer as there was no biotin band in this lane. These reactions were carried out at 37 °C for 2 h (B) The blot demonstrated that HsSIRT7 is not capable of transferring multiple units of ADP ribose. We have used the H187Y mutant and PARP1 as negative and positive control, respectively. The blot was developed with anti poly ADPr antibody (C) Chemical stability assay of auto-ADP ribosylation of HsSIRT7 showed hydrolysis of ADP-ribosyl linkage with both Arg and Cys residue. Purified HsSIRT7 was incubated with Bio-$NAD^+$ in the reaction buffer at 37 °C for 2 h. After that, the reaction mix was divided in equal aliquots and different hydrolyzing agents (0.5 mM) were added to it and kept for another 2 h at the same temperature. The reaction was stopped by adding sample dye. The reaction mixture was resolved on SDS PAGE gel and further immunoblot was carried to detect the presence of biotin (D) ADPr transfer reaction in presence of HsSIRT6: Here HsSIRT7 and H187Y mutant were used as substrates for ADPr transfer reaction in presence of HsSIRT6. This western blot was performed to check for the non-specific binding of ADPr with HsSIRT7. HsSIRT7 which has the ability to self ADP ribosylate, we see a biotin band in that lane. The H187Y mutant, which cannot hydrolyse $NAD^+$, did not show a non-specific binding of ADPr moiety with the mutant. Thus, HsSIRT6 is unable to transfer the ADPr moiety on the mutant. Similar experiments were also carried out using OsSRT1.

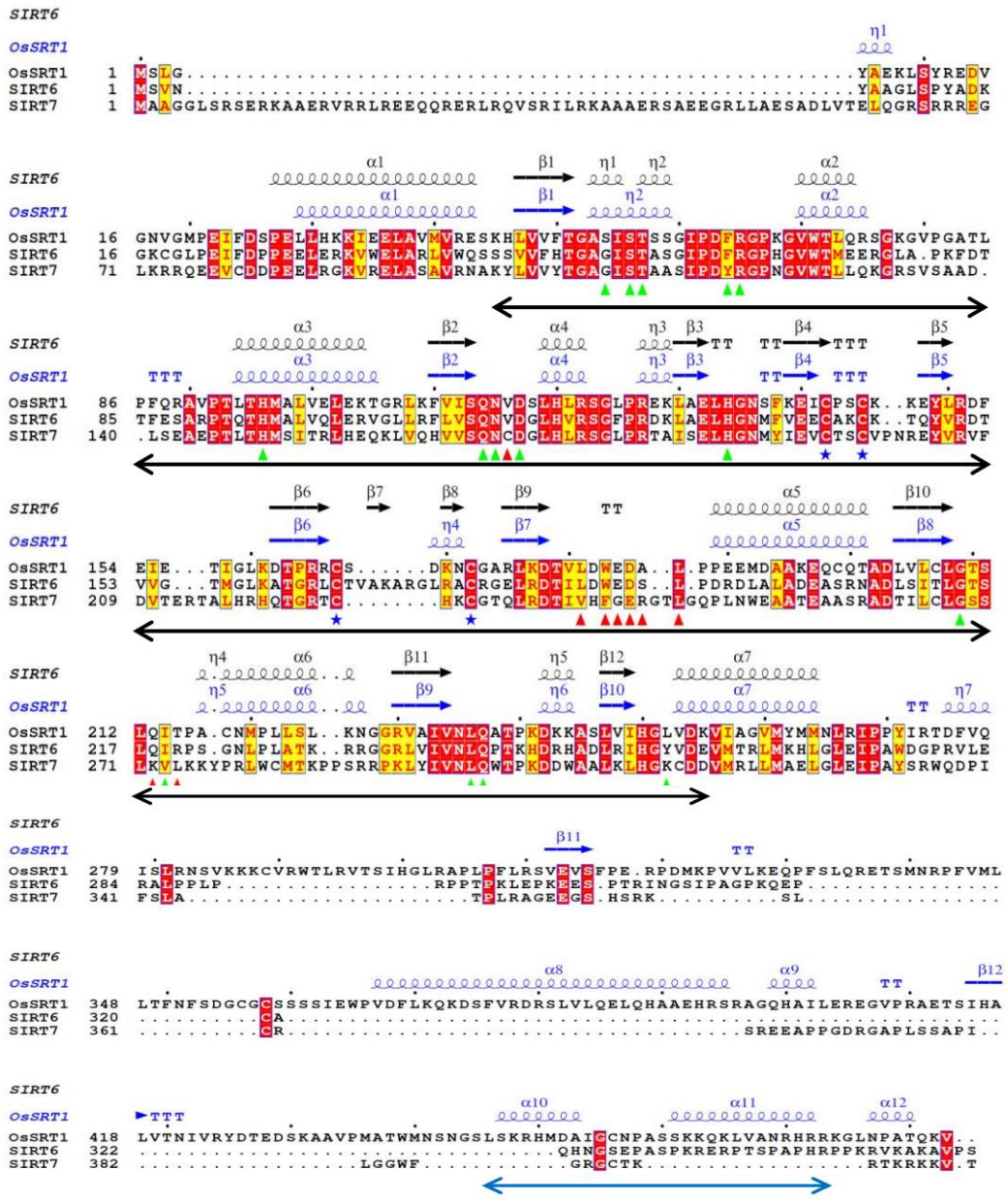



**Figure 2: Sequence alignment of class IV sirtuin members:**

Sequence analysis of class IV sirtuin members: Sequence alignment of OsSRT1 (B8ARK7) with its human homolog SIRT6 (Q8N6T7) and SIRT7 (Q9NRC8). The residues which line the $NAD^+$ binding region are shown in green triangles, peptide binding residues with red triangles, cysteines involved in zinc binding are shown with blue stars. The underlying black arrow demarcates the catalytic core region. The secondary structure depiction is based on the homology model coordinates. The underlined blue arrow at the C-terminus depicts the NLS based on cNLS mapper analysis. The figure is prepared using ESPript 3.0.

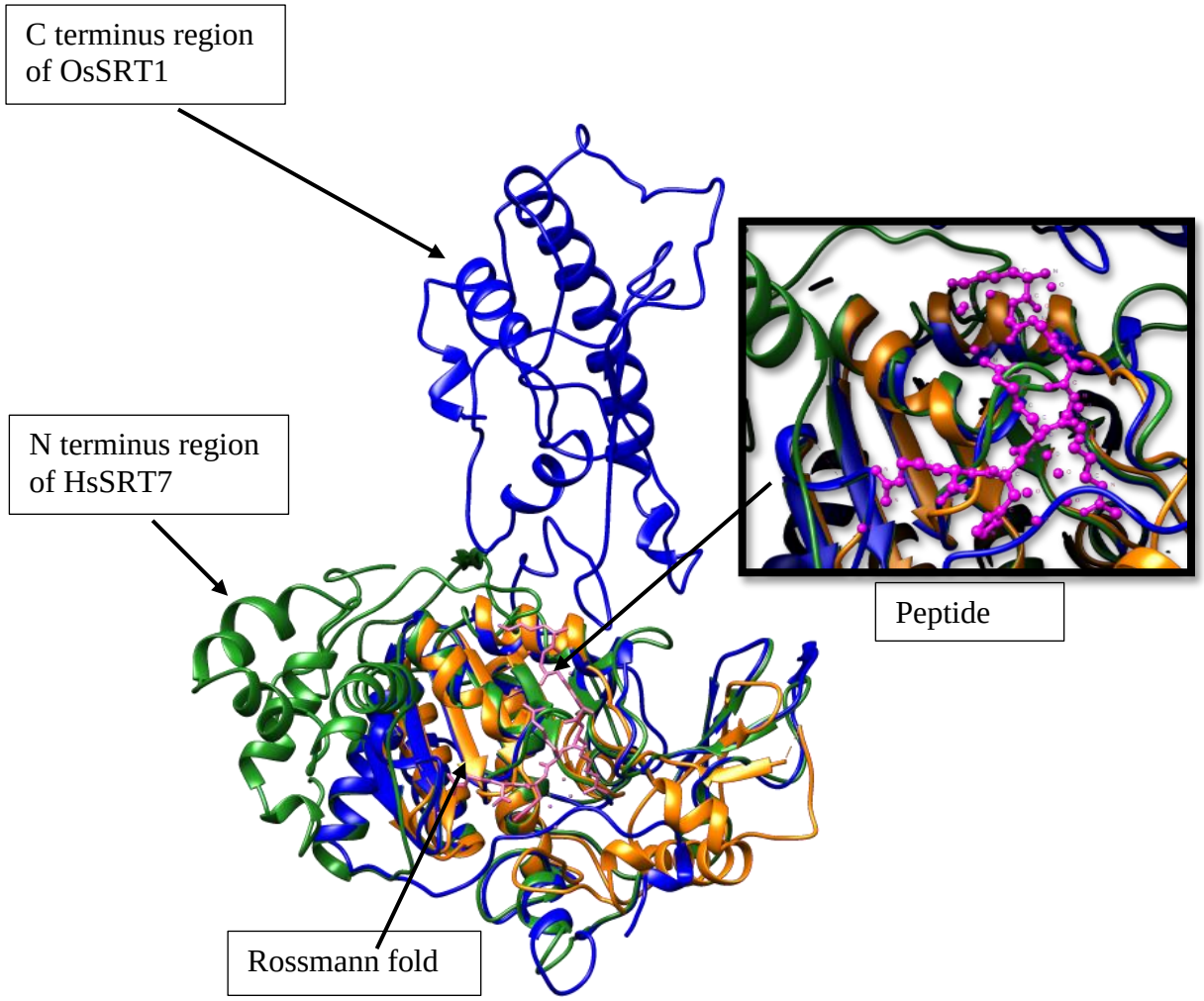



**Figure 3: Overlay of three-dimensional structures of class IV sirtuins members:**

Figure shows the cartoon depiction of the HsSIRT6, OsSRT1, HsSIRT7 models overlapping each other to understand the structural difference between class IV sirtuins. It clearly shows the two adjacent regions where the two ligands bind at the active site. Away from the active site, $Zn^{2+}$ is present in coordination with four Cys residues. From this structure, we can see the extended C terminus region of OsSRT1. Forest Green model is HsSIRT7, Blue Model is OsSRT1 Orange model is reference model HsSIRT6 (3pki), Structure is also docked with a peptide (Pink) to display the active site of the protein.

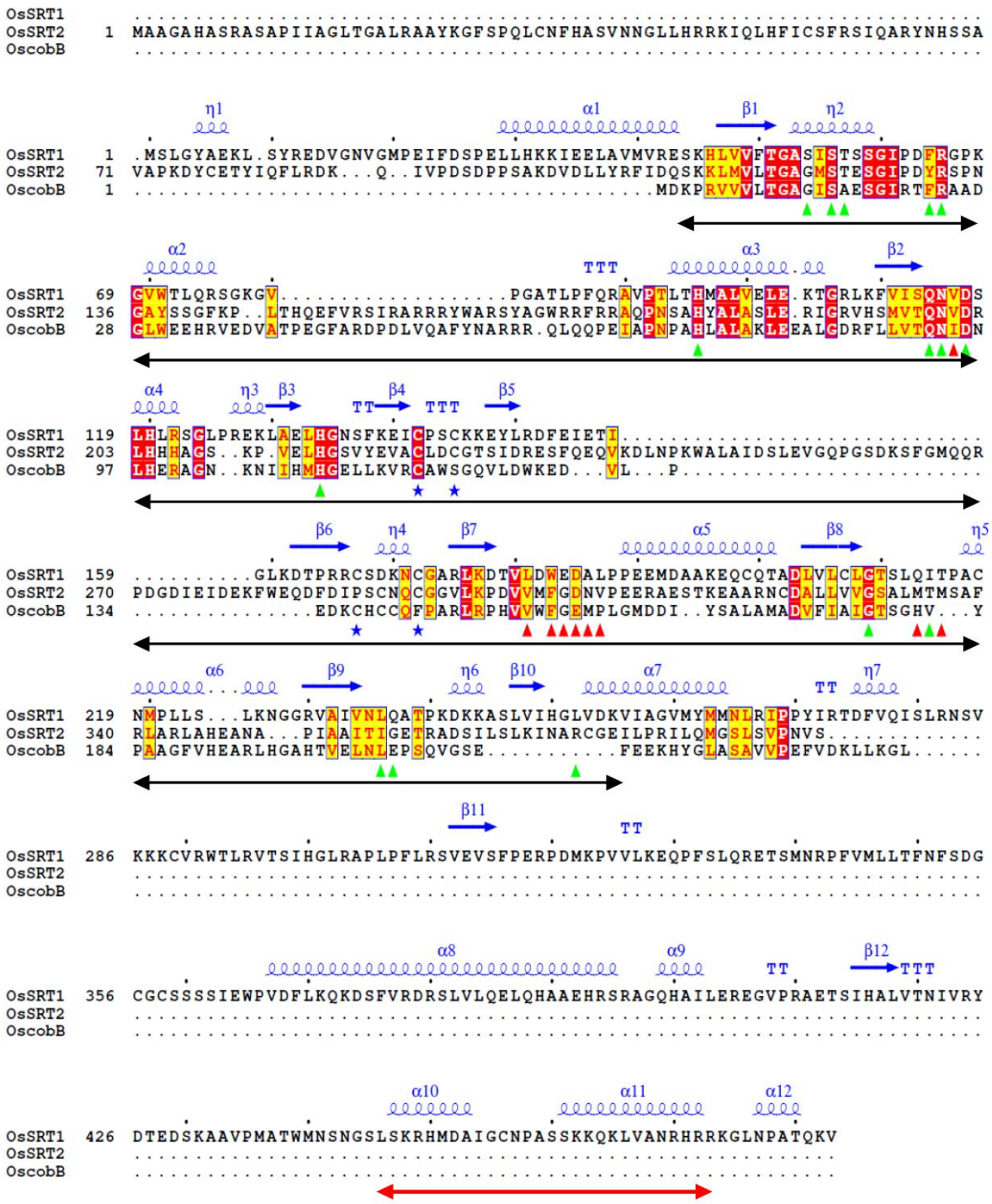



**Figure 4: Sequence alignment of plant sirtuins:** Sequence analysis of rice sirtuins: Sequence alignment of OsSRT1 (B8ARK7), OsSIRT2 (B8BNG4) and OscobB (A2XBC4). The residues which line the $NAD^+$ binding region are shown in green triangles, peptide binding residues with red triangles, cysteines involved in zinc binding are shown with blue stars. Black arrow demarcates the catalytic core region. The secondary structure depiction is based on the homology model coordinates. The underlined red arrow at the C-terminus depicts the NLS based on cNLS mapper analysis. The figure is prepared using ESPript 3.0.

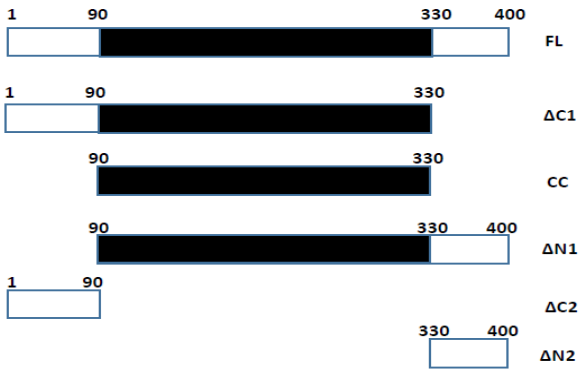



**Figure 5: Schematic representation of HsSRT7 constructs:** This figure represents various lengths of different HsSIRT7 counter parts. Full length protein represents as FL, construct without a C-terminus region represents as ΔC1, catalytic core represents as CC, construct without an N-terminus represents as ΔN1, N-terminus without other parts represents as ΔC2 and only C-terminus construct represents as ΔN2.

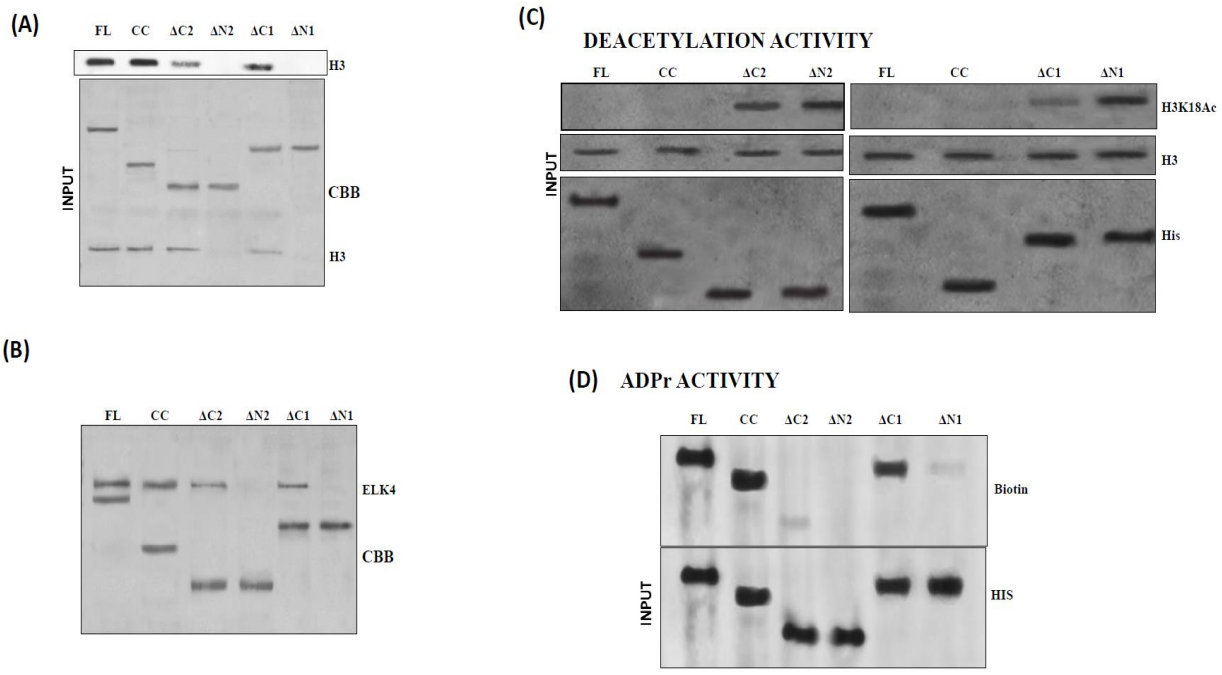



**Figure 6: Role of N and C terminus:**
Ni pull down assay was performed to study interaction of different constructs of His tagged HsSIRT7 with ligands. **(A)** Histone H3 and **(B)** ELK4. The resultant protein complexes pulled down by Ni-NTA beads were resolved on SDS-PAGE and presence of H3 in these complexes was further detected by western blot using anti H3 antibody. The coomassie brilliant blue (CBB) gel is shown in the bottom. For ELK4 complex studies, CBB stained SDS-PAGE gel showed the final complex bound to the Ni-NTA beads. This gel revealed the constructs which interacted with ELK4. Here, ELK4 alone did not non-specifically bind to the beads. **(C)**Anti-acetyl H3K18 western blot showing the ability of different HsSIRT7 deletion constructs to deacetylate H3K18Ac of H3 protein. **(D)** Anti-biotin western blot used to detect auto-ADPr transfer activity of full length HsSIRT7 and its truncations. The substrate used here is Bio-$NAD^+$ (40μM). The reaction was carried out at 37°C for 2 hr. The loading control is shown in the lower panel of the western blots.

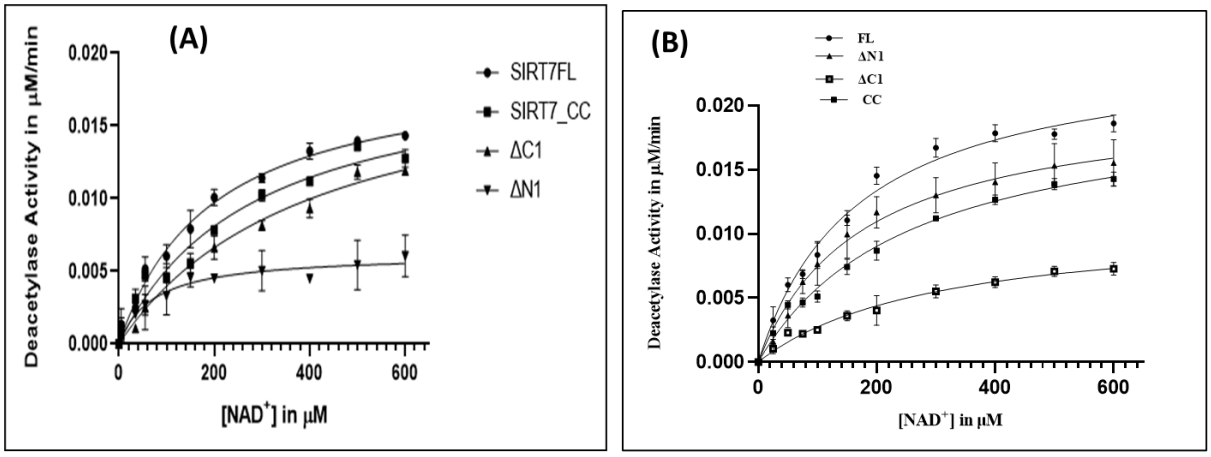



**Figure7: Saturation kinetics of class IV sirtuins: (A)** Saturation kinetics for the HsSIRT7 deacetylase activity: Deacetylation of H3K18Ac using varied concentrations of $NAD^+$(0-600 µM). The plot was calculated using Graphpad Prism 8.3.0. The error bar depicts the S.D.; n=3. **(B)** Saturation kinetics for the deacetylase activity of OsSRT1 constructs: Deacetylation of H3K9Ac using varied concentrations of $NAD^+$(0-600 µM). The plot was calculated using Graphpad Prism 9.1.1. The error bar depicts the S.D.; n=3.

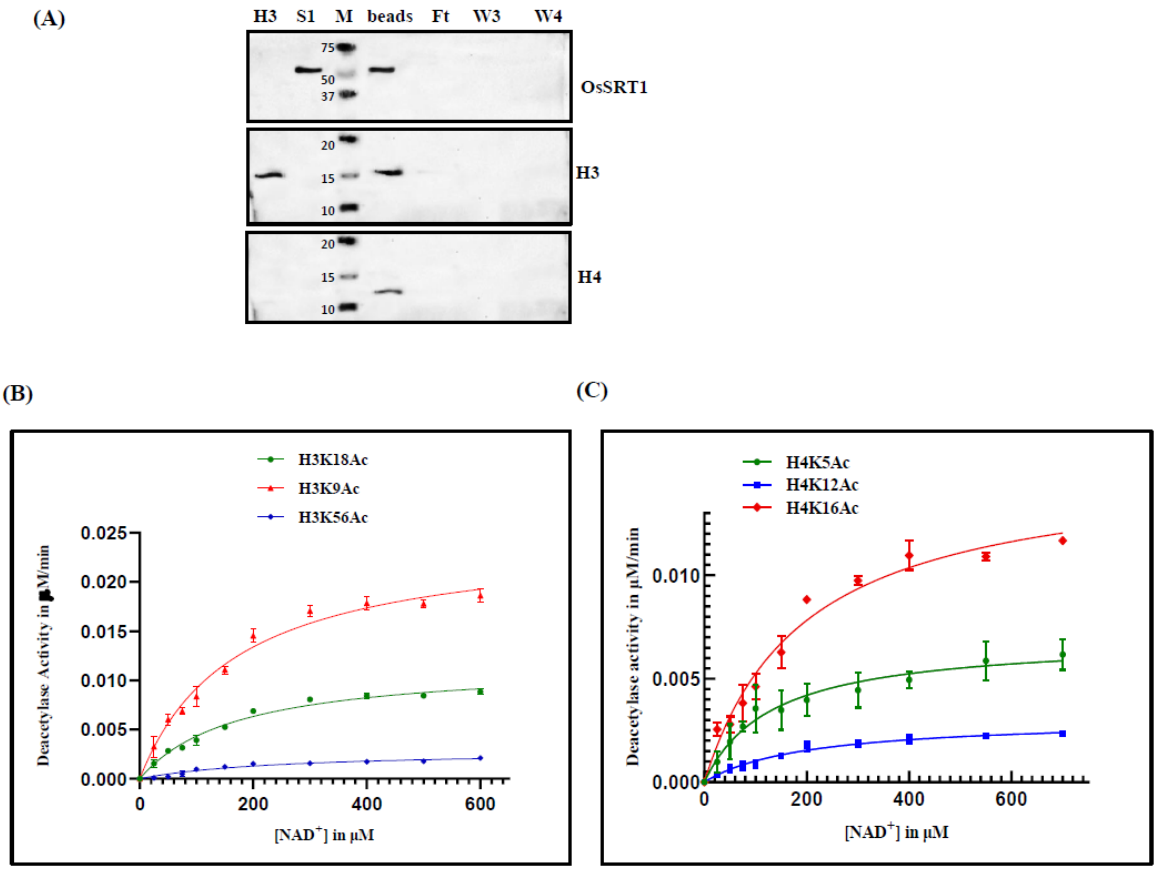



**Figure8: Histone deacetylase activity by OsSRT1:** (A) Interaction of OsSRT1 with histones H3 and H3 using Ni pull down experiments. This was performed to detect the interaction of His-tagged OsSRT1 with histones extracted from the nuclear fractions of rice leaves. After several wash steps, the resultant protein complexes pulled down by Ni-NTA beads were resolved on 12% SDS-PAGE. The presence of H3 and H4 in these complexes were detected by histone specific western blot. Initial flowthrough (Ft) and final washes (W3 and W4) of these experiments were also run on the gel. (B) Michaelis Menten kinetics for the OsSRT1 deacetylase activity: Deacetylation of different acetylated Lysine positions of H3 using varied concentrations of $NAD^+$ (0-600 µM). The plot was calculated using Graphpad Prism 9.1.1. The error bar depicts the S.D.; n=3. (C) Saturation kinetics for the deacetylase activity of OsSRT1: Deacetylation of the different acetylated lysine positions of H4 using varied concentrations of $NAD^+$ (0-600 µM). The plot was calculated using Graphpad Prism 9.1.1. The error bar depicts the S.D.; n=3.

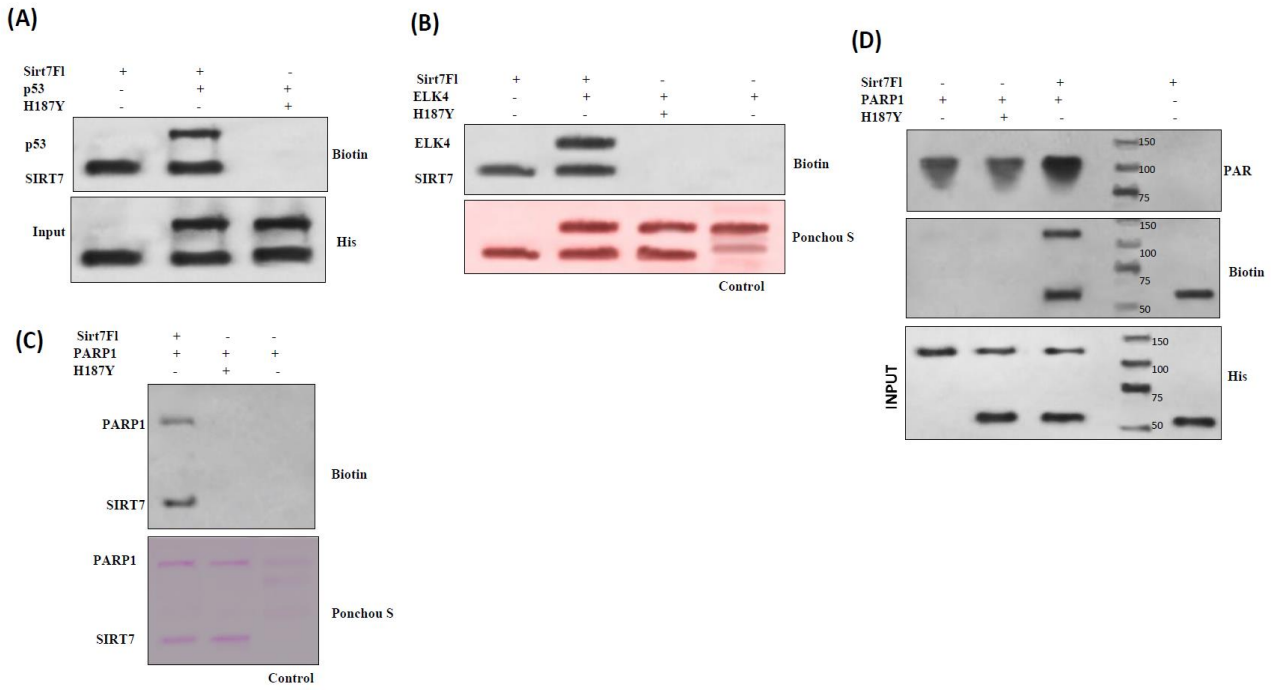



**Figure9: Interaction of HsSIRT7 with non-histone targets:**

Recombinant HsSIRT7 is able to transfer the biotinylated ADP-ribose on proteins (A) p53 (B) ELK4 (C) PARP1. The specific recombinant protein was incubated with HsSIRT7 in an ADP ribosylation reaction mix containing Bio-$NAD^+$ for 2 h at 37 °C. The reaction was stopped by adding sample dye, boiled and resolved on SDS-PAGE. It was then transferred on nitrocellulose membrane. The blot was probed with anti-biotin antibody to detect the presence of ADP ribose on respective proteins. Here the negative control is the empty vector or H187Y mutant. The loading control is shown in the bottom panel with anti His-tag blot or ponceauS staining (D) Regulation of PARP1 activity by HsSIRT7 mono-ADP ribosylation: Western blot analysis using poly ADPr antibody showed that in presence of HsSIRT7, the PARP1 activity increases. Addition of H187Y did not modify the PARP1 activity as shown in lane2.

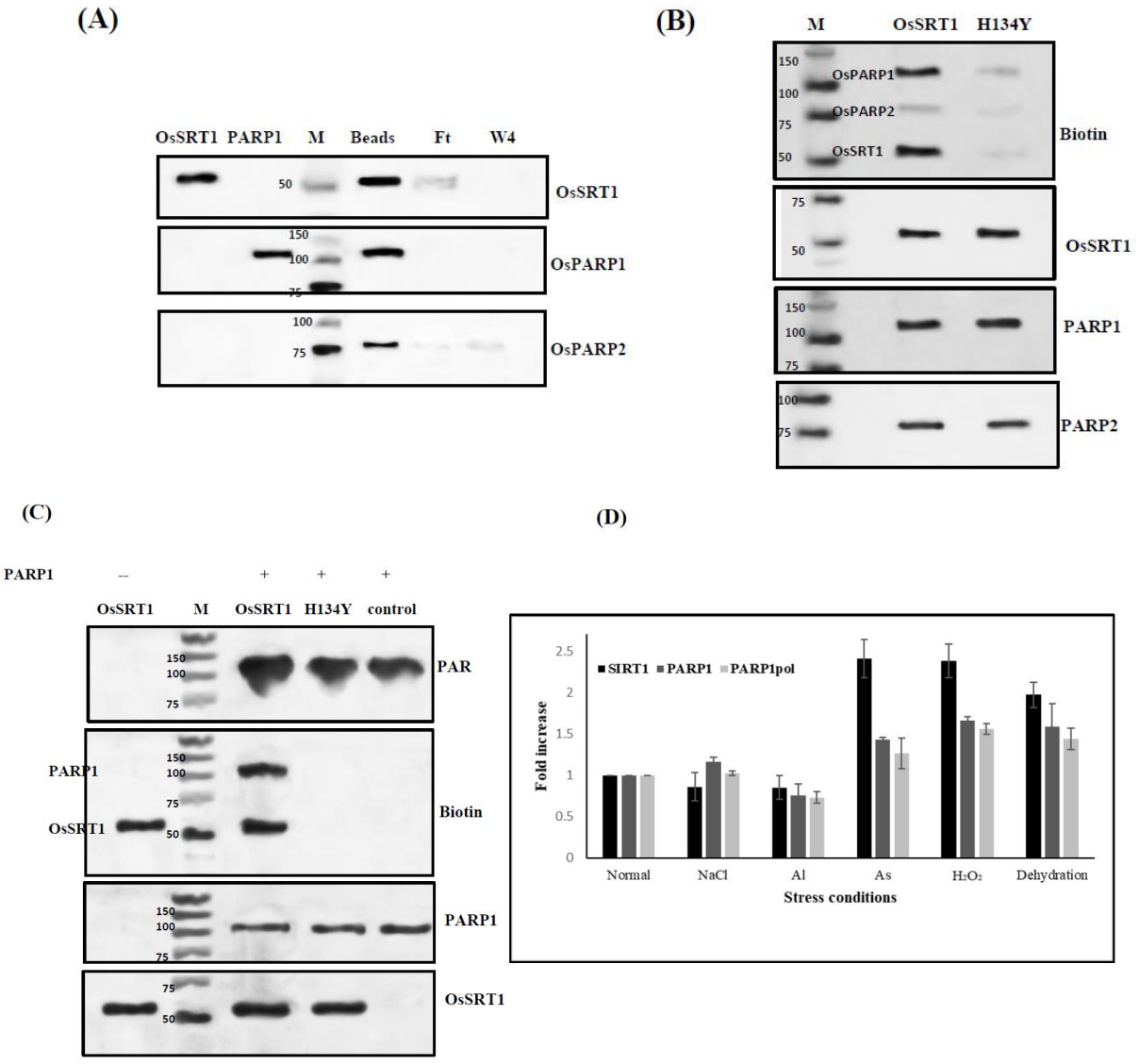



**Figure10: Interaction of OsSRT1 with non-histone targets: (A) Detection of interacting partners in nuclear extract of rice leaves.** Ni pull down experiment of recombinant HIS-OsSRT1 with nuclear extract of rice leaves showed the interactions with OsPARP1 and OsPARP2 as detected by protein specific antibodies. **(B) Plant PARPs are targets of ADP ribosylation by OsSRT1:** Recombinant OsSRT1 is able to transfer the biotinylated ADP-ribose on OsPARP1 and OsPARP2. The nuclear extract was incubated with recombinant OsSRT1 in an ADP ribosylation reaction mix containing Bio-$NAD^+$ for 1 hr at 25°C. The reaction was stopped by adding sample dye, boiled and resolved on SDS-PAGE. It was then transferred on nitrocellulose membrane. The blot was probed with anti-biotin antibody to detect the presence of ADP ribose on respective proteins. Here the negative control is the empty vector or H134Y mutant. The loading control is shown in the bottom panel with blots developed with

protein specific antibodies. **(C) Effect of OsSRT1 ADP ribosylation on PARP1 activity:** Western blot analysis using poly ADPr antibody showed that in presence of OsSRT1, the PARP1 activity increases. Addition of mutant H134Y or empty vector (control) did not modify the PARP1 activity as shown in lane 4 and 5. **(D) Effect of OsSRT1 upregulation on OsPARP1 activity:** The nuclear protein extract was prepared from the leaves of the rice plants exposed to the different abiotic stress conditions as mentioned in the figure. Western blot analysis was carried out using the specific antibodies (OsSRT1, PARP1 and PAR). All the data were normalized before plotting. Histone H4 in the nuclear extract was used as the internal control to normalize the signal. The histogram suggests that the PARP1 enzyme activity increased with increase in the expression of OsSRT1 under Arsenic, $H_2O_2$, and dehydration. It is possible that the OsSRT1 ADP ribosylates OsPARP1 to increase its activity.

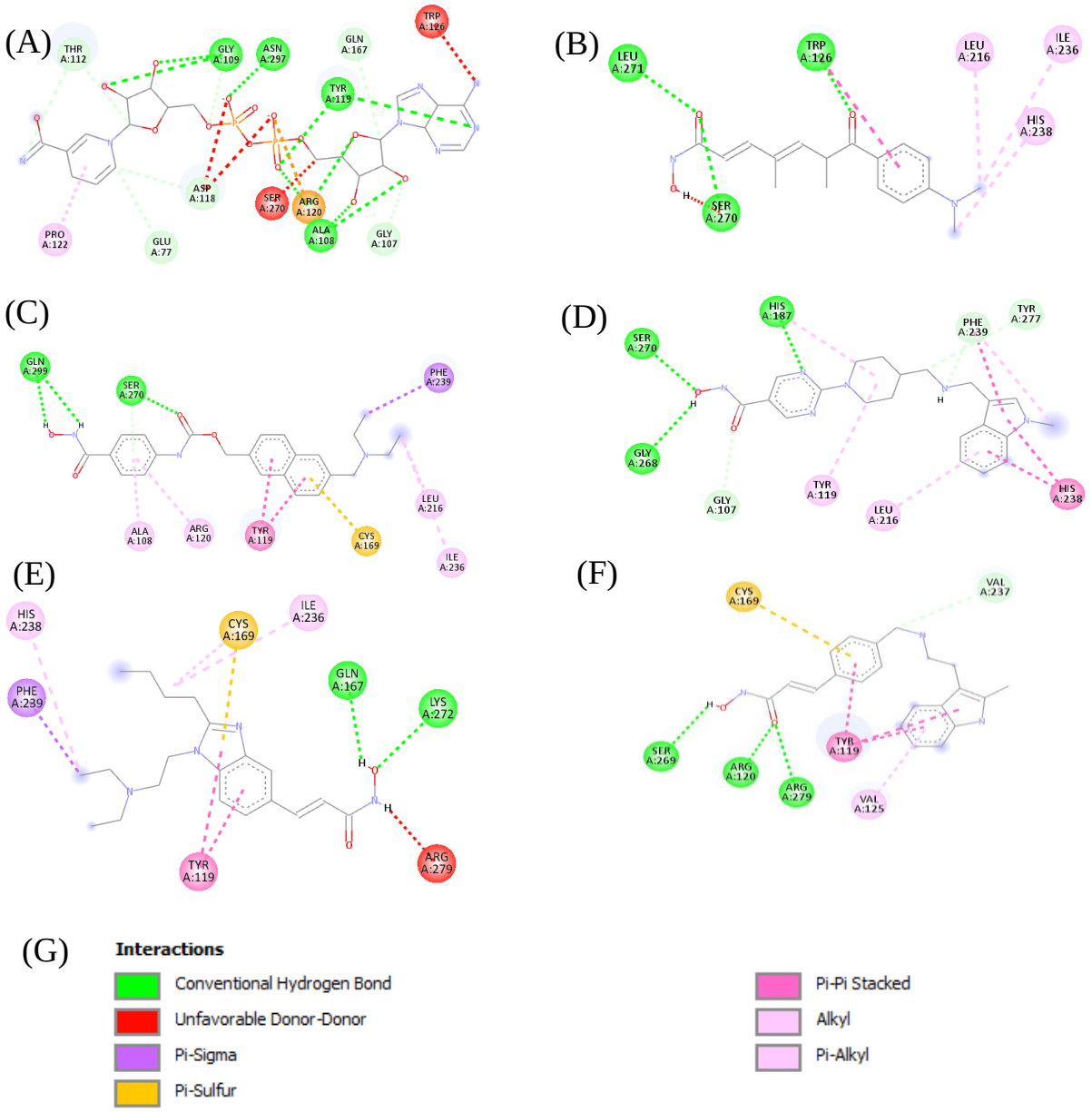



**Figure 11: Docking analysis of compounds against HsSIRT7 active site:** experiment has been done using PyRx- Virtual screening tool, and 2D images have been developed using Discovery Studio Visualizer. **(A)** NAD-HsSIRT7 interaction **(B)** PANOBINOSTAT (LBH589) interacts with HsSIRT7 **(C)** PRACINOSTAT (SB939) interacts with HsSIRT7 **(D)** TRICHOSTATIN A interacts with HsSIRT7 **(F)** DACINOSTAT (LAQ824) interacts with HsSIRT7 **(E)** SODIUM BUTYRATE interacts with HsSIRT7 **(G)** Index.

**Appendix:**
**List of Primers:**

| Number | F/R | Primer name | Sequence |
|---|---|---|---|
| **1** | F | OsSRT1_BamHI | 5' CGG GAT CCA TGT CAC TTG GCT ATG C 3' |
| **2** | R | OsSRT1_EcoRI | 5' CCG GAA TTC TCA AAC TTT CTG AGT AG 3' |
| **3** | F | OsSRT1_cc_NcoI | 5'CAT GCC ATG GTG CGA GAG AGT AAG 3' |
| | R | OsSRT1_cc_EcoRI | 5' CCG GAA TTC TCA CTT ATC CAC AAG TC 3' |
| **4** | F | OsSRT1_C_BamHI | 5'- CGC GGA TCC ATG GTT ATT GCG GGC GTT ATG TAC -3' |
| **5** | R | OsSRT1_N_EcoRI | 5'- CCG GAA TTC TCA GCT CTC ACG AAC CAT CAC -3' |
| **6** | F | T7 promoter_long | 5' CGA AAT TAA TAC GAC TCA CTA TAG GGG AAT TG 3' |
| **7** | F | Sirt6_BamHI | 5' CGC GGA TCC ATG TCG GTG AAT TAC GCG GCG 3' |
| **8** | R | Sirt6_EcoRI | 5' CGG AAT TCT CAG CTG GGG ACC GCC TTG GCC T 3' |
| **9** | F | Sirt7_CC_NcoI | 5' CAT GCC ATG GAG CTG GCC AGC GCC GTC 3' |
| **10** | R | Sirt7_CC_EcoRI | 5' CGG AAT TCT CAG GGG ATC TCC AAG CCC AGC T 3' |
| **11** | F | Sirt7_N1_NcoI | 5' CAT GCC ATG GCA GCC GGG GGT CTG AG 3' |
| **12** | R | Sirt7_C1_EcoRI | 5' CCG GAA TTC TTA GCA GCC CCT GCC AAA CCA 3' |
| **13** | F | SIRT7_S111Y | 5’ ACA GGC GCG GGA ATC TAC ACG GCA GCG TCT ATC 3’ |
| **14** | R | SIRT7_S111Y | 5’ GAT AGA CGC TGC CGT GTA GAT TCC CGC GCC TGT 3’ |
| **15** | F | SIRT7_S115A | 5’ ATC AGC ACG GCA GCG GCT ATC CCA GAC TAC CGG 3’ |
| **16** | R | SIRT7_S115A | 5’ CCG GTA GTC TGG GAT AGC CGC TGC CGT GCT GAT 3’ |
| **17** | F | SIRT7_R120A | 5’ TCT ATC CCA GAC TAC GCG GGC CCT AAT GGA GTG 3’ |
| **18** | R | SIRT7_R120A | 5’ CAC TCC ATT AGG GCC CGC GTA GTC TGG GAT AGA 3’ |

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**A) International Conference on Informatics & Integrative Biology 2014:**

International Conference on Informatics & Integrative Biology

December 17 - 19, 2014

Bose Institute, Kolkata

(CIIB-2014)

Certified that

Nilabhra Mitra

has participated in the International Conference on Informatics & Integrative Biology (CIIB-2014) Organized by the Bioinformatics Center, Bose Institute, Kolkata during 17 - 19 December, 2014

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Chairman
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Sudipto Saha
Joint Secretary
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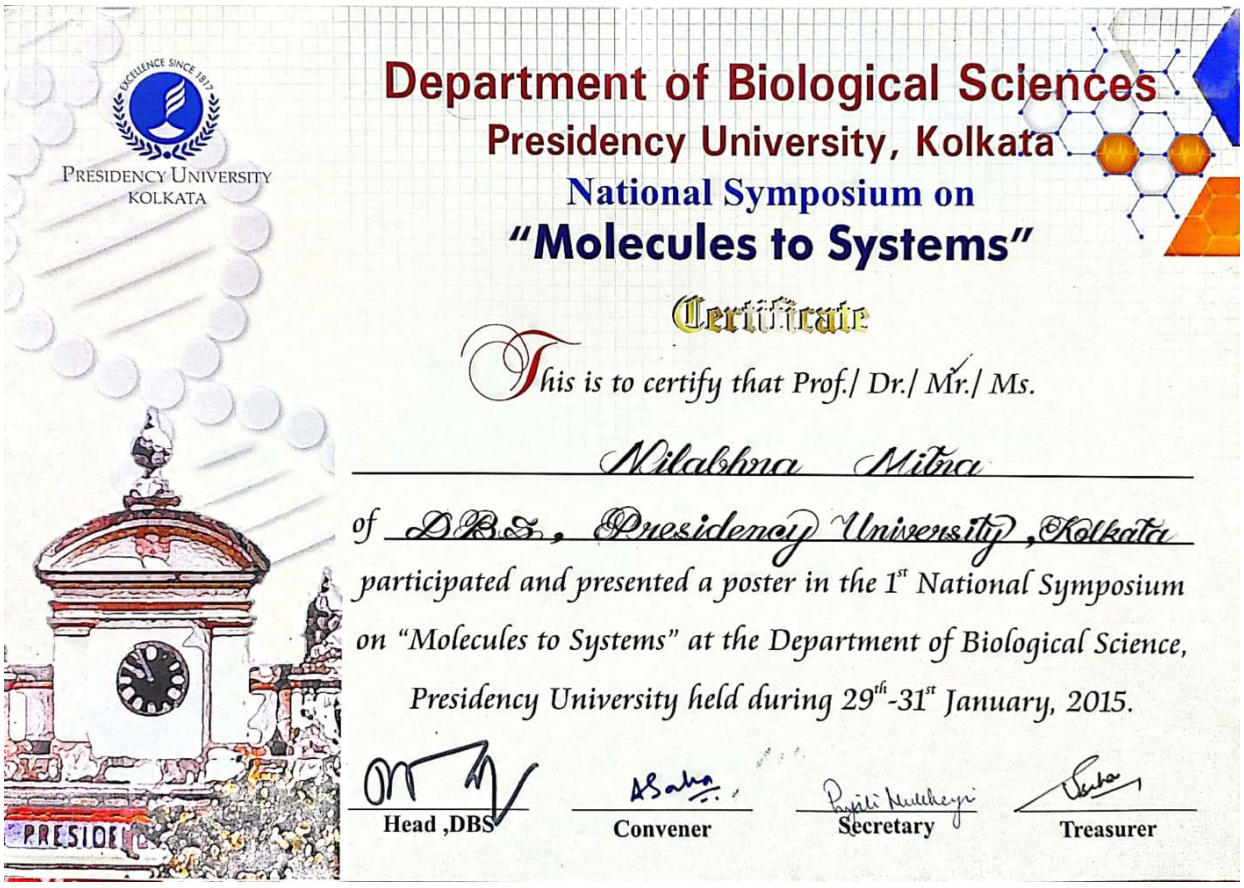

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23rd
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28–29 FEBRUARY, 2016

This is to certify that Prof/Dr/Mr/Mrs Nilabhra Mitra
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and/or exhibited model at the **23rd West Bengal State Science and Technology Congress, 2016**
held during 28–29 February, 2016.
Title of the paper/model/exhibit An Enzyme named Sirtuin, A Molecular Regulator.

**Upal Chakrabarti**
*Joint Organising Secretary*

**Soumendu Chatterjee**
*Joint Organising Secretary*

**C) National Virtual Conference (NVC-2021) on Genomics to Phenomics: A New Horizon 2021:**

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Prof. Binay Chaubey
Head, Department of Botany,
University of Calcutta

Dr. Maumita Bandyopadhyay, Dr. Sudipta Ray, Dr. Soumitra Paul
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# Biochemical characterization of mono ADP ribosyl transferase activity of human sirtuin SIRT7 and its regulation

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Transcription factor

ABSTRACT

SIRT7, an epigenetic modulator is related to several important cellular processes like aging, genome stability, and metabolism. The mechanistic and regulatory aspect of this enzyme needs to be explored. SIRT7 contains a conserved catalytic core with long flanking N- and C-terminal extensions. We find that the N terminus is involved in substrate binding, thus also in its dual enzyme activity i.e. deacetylation and ADP ribosylation. The C-terminus is not essential for its catalysis. Mutation of certain residues at the active site suggests that mono ADP-ribosylation and deacetylation are two distinct activities of SIRT7. In this study, we also find that the SIRT7 enzyme can specifically transfer a single moiety of ADP ribose on other nuclear proteins, with a preference for $NAD^+$. For this, the ADPr transfer follows the enzymatic reaction mechanism. Nicotinamide and certain metal ions have a significant negative effect on this mono ADP ribosylation process. A comparison of these dual activities suggests SIRT7's preference for the mono ADPr transfer over its deacetylation of H3K18Ac. Mono ADP ribosylation in cells is often linked to different metabolic disease conditions. This kind of modification of transcription factors, p53 and ELK4 by SIRT7 may play a key role in maintaining the tumor phenotype. Thus, SIRT7 becomes an important therapeutic hotspot for drug designing against several diseases. Finally, we can also relate SIRT7 to the DNA repair process through ADP ribosylation of one of its key players, PARP1. Here, SIRT7 positively regulates the PARP1 activity.

## 1. Introduction

Sirtuins are $NAD^+$ dependent deacetylases, divided into five subclasses: I-IV and U. Among the seven human sirtuins, SIRT1 to 3 belong to class I, SIRT4 and 5 belong to class II and III, SIRT6 and SIRT7 belong to class IV [1,2]. SIRT1, 6 and 7 are nuclear/nucleolar, SIRT3, 4 and 5 are mitochondrial and SIRT2 is mainly a cytosolic protein. The two members, SIRT6 and 7 share an overall sequence identity of approximately 38%. H3K9Ac and H3K56Ac are the substrates for SIRT6 deacetylation [3–5] but SIRT7 is very specific for H3K18Ac as a lysine deacetylase target [6]. The mechanism of this substrate specificity in SIRT7 is not quite clear. Three-dimensional structures of most of the human sirtuins are available in apo or/and different ligand complex forms. In the case of SIRT7, structural information of only the N terminal region (residue range 3–73) is available [7]. All the sirtuins have a phylogenetically conserved catalytic core and variable N and C terminal regions with respect to length and sequence [1]. The terminal regions of sirtuin proteins have varied functions. The N and C terminus of yeast Hst2 are involved in homotrimer formation, which is responsible for the autoregulation of its activity [8]. Among nuclear proteins, SIRT1 has extensions which regulate its deacetylase activity [9]. The rather shorter N-terminal end of SIRT6 is needed for its enzyme activity and C-terminus for subcellular localization [10]. SIRT7 has long N and C terminal regions whose roles are not well studied.

Sirtuins even though a member of the HDAC family are known to catalyse other reactions in addition to deacetylation. ADPr transfer activity was widely reported in sirtuins of several prokaryotes like *plasmodium falciparum* [11], *trypanosoma brucei* [12], *E. coli and salmonella* cobB [13,14] *etc*. Yeast Sir2 also possessed this dual enzyme activity [15]. Among human sirtuins, two of them were reported to have robust ADPr transfer activity. SIRT4, with very weak deacetylase activity, could downregulate glutamate dehydrogenase by ADPribosylating the enzyme [16]. In 2005, it was reported that mouse SIRT6 could self-ADP-ribosylate, but no deacetylation activity could be detected for this enzyme [17]. Further characterization of this enzyme mechanism was not carried out. Later, its weak deacetylase activity was reported [3,18]. Human SIRT6 could ADP ribosylate PARP1 and activate the DNA repair process [19]. It could also modify nuclear repressor

*Abbreviations:* ADPr, ADP ribose; HDAC, Histone deacetylase; PARP1, Poly ADP-ribose polymerase 1; KAP1, KRAB-associated protein 1

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